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THE INFLUENCE OF PLASMA PHOSPHATE ON THE PHOSPHATE CONCENTRATION OF HUMAN PAROTID SALIVA

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Abstract—Phosphate and hydrocortisone have been infused and glucose and sodium bicarbonate ingested in attempts to alter the plasma and salivary phosphate concentrations. After the phosphate and hydrocortisone infusions, the plasma phosphate concentration rose and fell respectively without significant changes in the salivary phosphate concentration. Glucose altered neither plasma nor salivary phosphate concentration. The salivary phosphate concentration was increased when sodium bicarbonate was ingested, which was probably due to the reduced rate of flow. The relationship between the composition and the rate of flow of saliva is discussed.

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IT HAS been reported (CLARKE and LEVINE, 1927; EDDY *et al.*, 1933; GURNEY and HUSCHART, 1950) that the oral administration of phosphates causes an increase, and the ingestion of carbohydrates causes a fall, in the salivary inorganic phosphate concentration.

The concentration of the electrolytes in saliva varies with the rate of its secretion (see Fig. 1; HILDES and FERGUSON, 1955; THAYSON, THORN and SCHWARTZ, 1956) and this must be taken into account in assessing the effect of experimental procedures. No mention is made of the rate of secretion in the published work concerning the oral administration of phosphates, and it is permissible to suppose that some variation in secretion rate may have arisen which could have caused the observed results.

In order to obviate changes in the rate of secretion, three methods have been used which will cause changes in the plasma phosphate concentration, but would not be

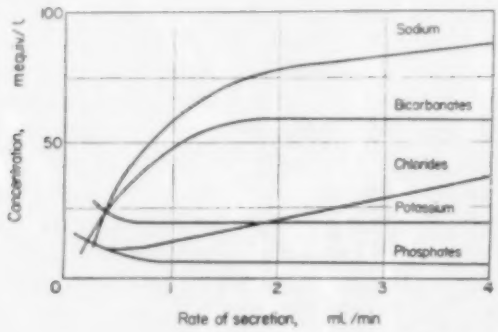

FIG. 1. The ionic concentration of saliva related to the rate of salivary flow. Values derived from HILDES and FERGUSON (1955) and THAYSON *et al.* (1956).

expected to change the rate of secretion of saliva. A fourth method has been used (ingestion of sodium bicarbonate) which causes a fall in the rate of salivary secretion and the results of these experiments can therefore be compared with those of the other three methods.

METHODS

The four methods used to attempt to change the plasma phosphate concentration are as follows:

1. *Phosphate infusions* (LONGSON *et al.*, 1956)

Buffered solutions which on analysis contained between 260 and 500 mM of phosphate (sodium dihydrogen phosphate 0·67% and disodium hydrogen phosphate 7·4% for a 500 mM solution) and had a pH range 7·0–7·4 were pumped into a forearm vein at the rate of approximately 3·6 ml/min from the start of the experiment. After 30 min a solution containing approximately 140 mM of phosphate (disodium hydrogen phosphate 2·46% and sodium dihydrogen phosphate 0·22%) was substituted, and the rate of infusion slowed.

2. *Hydrocortisone infusions* (MILLS and THOMAS, 1957)

100 mg of hydrocortisone free alcohol in 20 ml of 50% ethanol (Upjohn) was dissolved in 500 ml of either isotonic lactose or 5% dextrose and infused into an arm vein. The infusion was commenced at 1230 hours and was completed in 10 min.

3. *Glucose ingestion* (HARROP and BENEDICT, 1924)

Oral ingestion of 100 g of glucose in 100 ml of water at 1430 and 1515 hours.

4. *Sodium bicarbonate ingestion* (LONGSON and MILLS, 1953)

Oral ingestion of 10 g of bicarbonate in 100 ml of water at 1430, 1515, 1545 and 1615 hours.

Parotid saliva was collected by means of a modified Lashley cup (LASHLEY, 1916) from the right gland. The collecting device was positioned and a piece of wax (3 g approx., m.p. 49°C) was chewed on the right side. Since the rate of secretion depends on the degree of mechanical stimulation, the consistency and amount of wax was standardized and the subject instructed to chew at a constant rate throughout. After the saliva had been flowing for 1 min the collection was made into a weighed test-tube over a timed period. Re-weighing enabled the rate of secretion in grammes per minute to be calculated. Saliva samples were collected at 1430 hours and every 30 min to 1630 hours. Blood samples were obtained from an indwelling Guest cannula in a forearm vein. In the phosphate infusion experiments two cannulae were used, one in each arm, whilst in all the other experiments, including the control, only one was inserted, for the purpose of removal of blood or infusion of the hydrocortisone.

Analytical methods

The FISKE and SUBBARROW (1925) method was used for the estimation of the inorganic phosphate. Variations from the usual procedures were as follows:

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Saliva. 0·5 ml of saliva was added to 8 ml of 10% trichloracetic acid and 1·5 ml of distilled water added to make the volume to 10 ml. The routine procedure was carried out on this sample.

Blood. In cases where the plasma phosphate concentrations were above the normal level only 3 ml of trichloracetic acid filtrate was used and the molybdate solution was made up of six parts of "molybdate 2" and four parts of "molybdate 1" in order to compensate for the reduced amount of acid filtrate, thus maintaining the correct pH when the volume was made up to 10 ml.

All experimental results are means of duplicates and the phosphate is recorded in mM concentration. The optical densities of the solutions were measured on a Hilger colorimeter (model H 810) with a Kodak filter (610 mμ), and a calibration curve was produced for each experiment using standard phosphate solutions.

Statistical analysis

The significance of differences between means was estimated by the standard small sample technique of FISHER (1941), on the results which were obtained at each 30 min interval.

EXPERIMENTAL

The FISKE and SUBBARROW method, like all those colorimetric techniques employing the formation of phosphomolybdic acid to estimate the salivary phosphate, suffers from the disadvantage that in certain subjects the trichloracetic acid filtrate becomes turbid when the molybdate is added, and seems to clear to a certain extent on addition of the aminonaphthol–sulphonic acid. The cause of this turbidity has not been established but it was found (WAINWRIGHT, 1938) that eliminating the turbidity by centrifuging or filtering led to a loss of phosphorus. WAINWRIGHT has investigated the colorimetric methods for the estimation of phosphorus and obtained satisfactory results with several, including the FISKE and SUBBARROW method. Since this method has been proved for the estimation of blood phosphates it was adopted for estimations on both fluids. Whilst it was felt that clear solutions should ideally be used for colorimetric methods, it was not known how many subjects would exhibit this feature of turbidity and to what extent this would mask the large change in phosphate concentration expected (CLARKE and LEVINE, 1927).

In order to assess the degree of turbidity the routine procedure for phosphorus determinations was carried out excluding the aminonaphthol–sulphonic acid reagent. The optical density of this turbid solution was then estimated using the normal procedure. Repeated estimations of the turbidity of the saliva from one subject indicated that it remained relatively constant and represented about 10 per cent of the total optical density recorded in the normal procedure. In order to find out if the turbidity would mask small changes in the phosphate concentration, 0·5 ml of saliva, exhibiting this feature, was added to each of six test-tubes containing 8 ml of 10% trichloracetic acid. The contents of each were then made up to 10 ml with phosphate solutions such that the amount of phosphorus varied in each tube. When the phosphate contents were estimated, the amount recovered could be ascertained. The results are shown in

Table 1. These results suggest that the turbidity does not interfere with accurate estimation of phosphate concentration. Repeated estimates (nine) of phosphate concentration on one sample of saliva gave a standard deviation of ± 0·06 mM on a mean value of 3·82 mM. Calculation of the standard error (Mainland, 1938) from fifty paired analyses gave a figure of ± 0·16 mM which represents an error of 2 per cent on the higher experimental results (8·0 mM) or 4 per cent on the lower ones.

Table 1. Addition of various amounts of phosphates to samples of a saliva producing turbidity and their recovery as estimated by the Fiske and Subbarrow method

Tube	Phosphorus (mg)					
	a Saliva	*b* Amount added	*c* Found	*d* Recovered ($c-a$)	*e* Difference ($d-b$)	% Recovery ($d/b \times 100$)
1	0·049	0	0·049	—	—	
2	0·049	0·004	0·0525	0·0035	−0·0005	87
3	0·049	0·012	0·059	0·010	−0·002	83
4	0·049	0·020	0·0675	0·0185	−0·0015	92
5	0·049	0·024	0·073	0·024	0·000	100
6	0·049	0·028	0·079	0·030	+0·002	107

RESULTS

The four types of experiment were repeated five times on subject JFB and the results of these experiments are recorded in Fig. 2 together with a control series. In nine control experiments the salivary phosphate concentration remained relatively constant over the whole period with a mean of 5·8 ± 0·6 mM. The plasma phosphate concentration varied between 1·0 and 1·4 mM.

Phosphate infusion

The plasma phosphate concentration rose rapidly by this method to 3·2 mM in the first 30 min and then fell slowly to 2·7 mM towards the end of the experiments. The rate of flow of saliva did not differ consistently from that in control experiments. The phosphate concentrations of the saliva were sometimes higher and sometimes lower than in the control experiments; the means of each half-hourly period after the start of the infusion were slightly above the control level, but even the largest difference of 0·8 mM in the second half-hourly period was not significant.

Hydrocortisone

The plasma phosphate concentration was reduced over the whole period of observation (mean 0·8 mM). Despite this the salivary phosphate concentration remained unaltered and the rate of flow of the saliva did not differ from the control.

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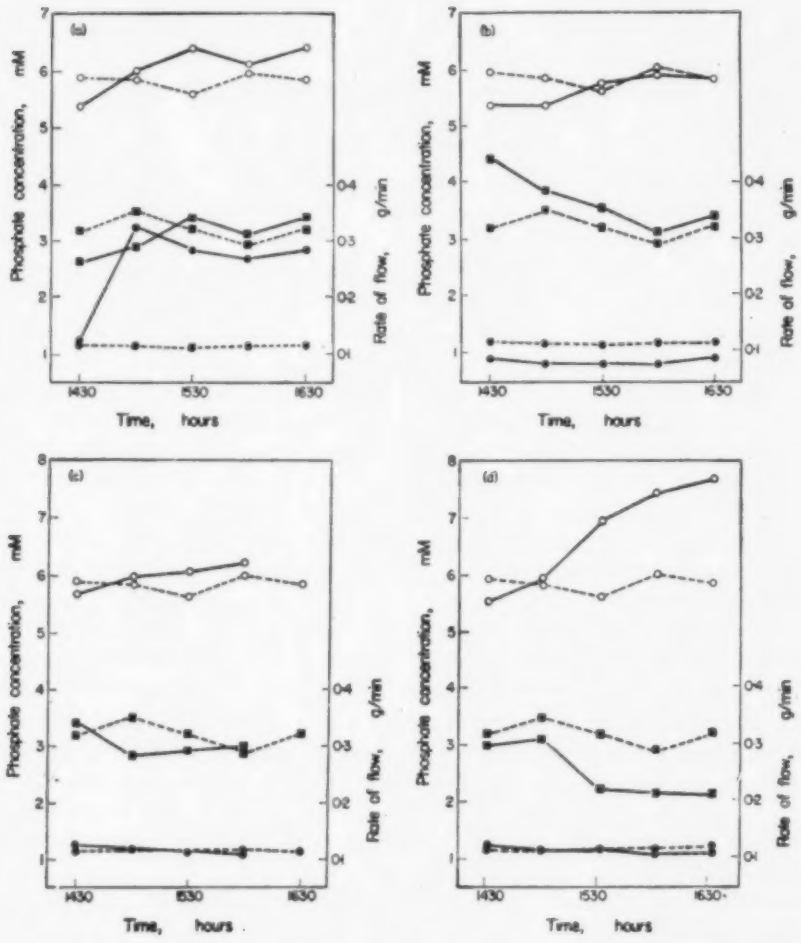

FIG. 2. The mean values of four sets of experiments (solid lines) on Subject JFB, together with a similar set of control results (broken lines). (a) Phosphate. (b) Hydrocortisone. (c) Glucose. (d) Bicarbonate. ■ Rate of flow. ● Plasma concentration. ○ Saliva concentration.

Glucose ingestion

There was no significant change from the control in any of the three variables studied. A single experiment of each sort and a control were performed on each of four further subjects. In all subjects the plasma phosphate concentration followed a similar pattern to that observed on JFB. As in JFB the salivary phosphate was slightly increased with the phosphate infusion, but unaltered by hydrocortisone or glucose.

Sodium bicarbonate ingestion

This was only attempted on JFB. The plasma phosphate concentration did not change significantly. A gradual increase in the salivary phosphate concentration was noted together with a reduction in the rate of flow over the period of observation.

DISCUSSION

The chemico-parasitic theory of MILLER (1890) concluded that acid-forming bacteria reduced the pH of the saliva in contact with the teeth to a sufficiently low level to cause solution of the inorganic constituents of the teeth. JENKINS (1954) says that a high buffering power of saliva may be an important factor in preventing such an attack and that the critical pH is governed by the calcium and phosphate concentrations of the saliva. Several workers (CLARKE and LEVINE, 1927; EDDY *et al.*, 1933; HAWKINS, 1931; GURNEY and HUSCHART, 1950) have attempted to show that the diet affects the phosphate concentration or the ingestion of phosphate will cause an increase in salivary phosphate concentration, and suggested this as a means of caries control. Since the increase in plasma phosphate in the experiments reported in this paper was large compared to the normal variations caused by dietary means, these claims must be viewed with grave doubt.

The ingestion of salts may cause a change in the rate of flow, as has been shown in the sodium bicarbonate experiments, and it is therefore of paramount importance that the rate of flow should be recorded in all experiments on salivary composition. This procedure does not appear to have been adopted by previous workers.

In the sodium bicarbonate experiments the fall in the rate of flow was only small (0·1 g/min) but at low rates of flow this is important. It has been impossible to interpolate from the work of HILDES and FERGUSON (1955) what effect this would have on the phosphate concentration of the saliva other than that an increase would take place (Fig. 1). This corresponds to the results obtained in the experiments reported here.

EDDY *et al.* (1933) claimed that carbohydrate in the diet reduced the salivary phosphate concentration. No qualification as to time of day, etc. was made and since it was known that ingested glucose (HARROP and BENEDICT, 1924) would produce a lowered plasma phosphate concentration in the morning, the experiment was repeated in the afternoon to see if any change in plasma or salivary phosphate could be produced.

It would be necessary to ingest between 2·2 and 3·1 g of phosphorus, allowing for a 70 per cent absorption (THORPE, 1952) to obtain an amount equal to that infused, and it would seem improbable that this would be so under normal dietary conditions. (This is equal to 1000 g brown bread, 6 pints of milk or 1500 g egg.)

Therefore, it would seem that any claim for changes in salivary phosphate due to diet should be accepted only if demonstrated directly by saliva analyses under known conditions of salivary flow rate.

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CHROMATOGRAPHIC STUDIES OF CARBOHYDRATE COMPONENTS IN ENAMEL

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Abstract—Enamel powder, purified by the Manly–Hodge procedure, was demineralized by dialysis against ethylene diamine tetracetate (pH 7·1) and the non-dialysable organic matter hydrolysed by incubation with Dowex 50- X12 and 0·05 N HCl at 105°C for 24 hr. The enamel hydrolysates were analysed for carbohydrate constituents by paper chromatography using mainly a pyridine: *n*-butanol: water solvent system (1:3:1). Six aldose sugars were recovered from hydrolysates of both human and bovine enamel organic matter. Three aldohexoses—galactose, glucose and mannose account for 1·55 mg of the 1·65 mg total aldose per 100 g enamel. The three other aldoses were tentatively identified as fucose, "rhamnose" and xylose.

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ENAMEL is a tissue of ectodermal origin containing approximately 0·2–0·4 per cent of organic matter. The major organic components are a protein which has been identified as an eukeratin (BLOCK, HORWITT and BOLLING, 1949; HESS and LEE, 1953; LOSEE and HESS, 1949; STACK, 1954) and an interprismatic ground substance of unknown composition (ORBAN, 1957).

Early histochemical studies of the organic components of enamel by WISLOCKI, SINGER and WALDO (1948) and WISLOCKI and SOGNNAES (1950) showed that adult human, guinea pig and rhesus monkey teeth exhibit metachromatic and basophilic staining in the organic matrix of enamel. The enamel matrix of developing rats' teeth is not metachromatic when initially formed, but only becomes so at a later stage. LISON (1936) and WISLOCKI, BUNTING and DEMPSEY (1947) stated that metachromasia is indicative of sulphated acid mucopolysaccharide. Such polysaccharides, composed mainly of hexuronic acid, hexosamine and sulphate, have been isolated from many body tissues including skin (PEARCE and WATSON, 1949), cornea, cartilage and bone (MEYER *et al.*, 1956) and dentine (HESS and LEE, 1952; KUMAMOTO, 1955). The possibility that sulphated acid mucopolysaccharide exists in enamel is heightened by the reports that enamel organic matter contains carbohydrates (PINCUS, 1948) and hexosamines (STACK, 1954). BELANGER (1955) has demonstrated the incorporation of radiosulphate into enamel matrix during maturation, the time when metachromasia is also exhibited.

In addition to acid mucopolysaccharides, many tissues, such as cornea and calf bone, also contain neutral mucopolysaccharides, which have aldose sugars—galactose, glucose, mannose and fucose as their principal carbohydrate constituents (GLEGG, EIDINGER and LEBLOND, 1953, 1954; KUMAMOTO, 1955; MEYER *et al.*, 1956; LEBLOND, GLEGG and EIDINGER, 1957). The carbohydrate–protein complexes, isolated from skin, lung, tendon, cartilage and bone, all give an intense periodic acid Schiff (PAS)

reaction *in vitro* and appear responsible for the positive PAS reaction of histologic sections from these tissues (LEBLOND *et al.*, 1957). Such neutral mucopolysaccharide may be present in enamel, since ENGEL (1948) and GREULICH and LEBLOND (1954) observed that developing enamel gives a positive PAS reaction. FULLMER and ALPHER (1958) indicated that the PAS reaction is much weaker in maturing enamel than in developing enamel. KUMAMOTO and LEBLOND (1958) reported that C^{14} labelled glucose or mannose is incorporated into the pre-enamel matrix of rats. The labelled material decreases in intensity, as the enamel mineralizes, until it can no longer be detected. Similarly, BELANGER (1955) reported that when radiosulphate (as $H_2S^{35}O_4$) is administered to rats, the radioactivity is soon detected in pre-enamel and young enamel, only to disappear gradually as the organic matrix calcifies. The substance is acid-fast and hyaluronidase-resistant. These results indicate that neutral mucopolysaccharide is present in developing enamel, but largely disappears as the enamel matures. Nevertheless, STACK (1954) and EGYEDI and STACK (1956) found that mature enamel contains "glucose" as part of a carbohydrate–protein complex.

The present report describes the isolation and identification of some carbohydrate components of enamel by ion exchange and paper chromatography methods.

METHODS AND MATERIALS

Preparation and purification of powdered enamel

Non-carious human and bovine teeth stored in 95% ethanol were used in this study. The teeth were cleansed by lightly grinding the external surface with a diamond stone to remove all visible stains, pit and fissure structures. The cleansed teeth were pulverized in a hammer mill and the powder that passed a 100-mesh sieve was purified by a modification of the flotation technique of MANLY and HODGE (1939). A bromoform–acetone mixture of specific gravity 2·75 was used for the first separation of enamel from dentine. Pure bromoform (s.g. 2·89) was used for subsequent flotations. The procedure was repeated four to eight times on the enamel fractions until the dentine impurities were reduced below 0·1 per cent "junction" particles. In the case of powdered bovine teeth, centrifugation (1250 r.c.f.) was necessary to accelerate the settling of enamel in the bromoform–acetone mixture.

Demineralization

From 7·5 to 15 g of purified enamel powder was placed in a cellophane dialysis sac; this was suspended with constant agitation in 1·5 l. of 0·25 M ethylene diamine tetracetate (pH 7·1) containing 0·012% "zephiran" chloride. Demineralization was usually complete in 7 days after which the dialysate was suspended in de-ionized water which was changed twice daily for 3 days to remove the decalcifying solution. The dialysing sac was presumed to contain only non-diffusible organic molecules. The contents of the sac were centrifuged (30,000 r.c.f. for 30 min) to separate the insoluble from the soluble fraction. The soluble fraction was passed through a sintered glass filter (ultrafine), evaporated to dryness *in vacuo*, and stored at −5°C.

Hydrolysis of "polysaccharides" in the enamel organic matter

The enamel organic matter (soluble or insoluble fraction) was transferred to a 2 ml glass ampoule containing 500 mg Dowex 50- X12 cation exchange resin and 1 ml of 0·05 N HCl. Hydrolysis was effected by heating the sealed ampoules at 105°C for 20–24 hr (ANASTASSIADIS and COMMON, 1955 ; BURGESS, 1958). The ampoule contents were transferred quantitatively to a 2 ml coarse, sintered glass filter, and the resin was washed with four 2 ml portions of water. The combined filtrate and water washings containing neutral sugars and uronic acids were lyophilized and stored at −5°C for subsequent chromatographic analysis. Hexosamines and amino acids remained adsorbed on the cation exchange resin; subsequently, the hexosamines were quantitatively eluted with 10 ml of 2 N HCl. The eluate was vacuum desiccated over sodium hydroxide pellets and stored at −5°C for subsequent analysis.

Chromatographic analysis

Filter paper (Whatman No. 3 MM, 18 × 22 in.) was washed according to the method of CONNELL, DIXON and HANES (1955). This procedure removed much impurity from the paper and permitted a sharper separation of sugar mixtures.

The enamel hydrolysate preparations containing the neutral sugars were redissolved in 0·04 ml water and transferred to the paper in 0·01 ml aliquots—each aliquot was allowed to dry before the next was applied. Standard sugar mixtures (50 γ and 100 γ of each sugar) were applied to adjacent positions on the same paper (see Fig. 1). After the initial descending development, the papergrams were dried and redeveloped in the same solvent to improve the separation of the sugar spots (JEANES, WISE and DIMLER, 1951). The following solvents were used in this study:

(1) pyridine: *n*-butanol: water (1:3:1 v/v); used for separating simple sugars,
(2) propionic acid: *n*-propanol: water (1:3:1 v/v); for separating hexuronic acids,
(3) 2% ammonium hydroxide: 2-butanol (1:4 v/v); for separating hexosamines.

Sugar spots were revealed by dipping the air-dried paper in a bath of aniline–hydrogen oxalate–acetone (aniline, 0·4% v/v; 1 M oxalic acid, 2% v/v; acetone, 97·6% v/v) followed by heating in an oven at 85°C for 20 min. The enamel sugar spots were tentatively identified by comparing their colour and R_f value with the colour and R_f of standard sugars on the same chromatograph. The approximate quantity of sugar in the hydrolysate spots was determined by comparing the spot density with that of the corresponding sugar standards.

RESULTS

A total of six different tooth samples (three bovine and three human) were analysed by the foregoing procedure. Each tooth sample was processed, from enamel purification to paper chromatography, at a different time, thereby reducing analytical bias.

Aldose sugars

Hydrolysates of the insoluble and soluble organic fractions from 7·5 g of bovine enamel were chromatographed in the pyridine solvent. The insoluble fraction (Fig. 1a) contained sugars which had R_f values similar to the standard sugars galactose,

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glucose, mannose, xylose, fucose and possibly rhamnose. (The sugar spots of fucose and rhamnose are not apparent in the photographic reproduction.) The soluble organic fraction (Fig. 1b) was qualitatively similar in its aldose sugar composition. In all six analyses, the quantity of sugars was less in the soluble fraction than in the insoluble fraction.

Human enamel displays the same aldose pattern: galactose, glucose and mannose with traces of xylose, fucose and possibly rhamnose in both the insoluble (Fig. 2a) and soluble (Fig. 2b) organic fractions. When chromatograms are developed in the pyridine solvent, it is difficult to distinguish between rhamnose and the lactone form of glucuronic acid since these sugars have a similar R_f value. With the propionic acid solvent, the lactone form of glucuronic acid does not occur; thus, the identification of rhamnose is more definite. The chromatograms developed in this solvent show an enamel sugar spot corresponding in position and colour to the rhamnose standard (Fig. 3).

TABLE 1. APPROXIMATE CONCENTRATION OF ALDOSE SUGARS IN ENAMEL

Species	No. of samples	Mg sugar per 100 g enamel powder						Total aldose
		Galactose	Glucose	Mannose	Fucose	Xylose	Rhamnose	
Human	3	0·83	0·63	0·23	0·04	0·05	Trace	1·78
Bovine	3	0·72	0·43	0·26	0·06	0·03	Trace	1.51
Average and standard deviation of six human and bovine samples		0·77 ±0·19	0·53 ±0·19	0·25 ±0·17	0·05 ±0·03	0·04 ±0·05	Trace	1·65 ±0·48
% of total aldose sugar		46·8	32·4	15·1	3·2	2·5	—	100

Quantitative data was obtained from paper chromatograms by comparing the densities of hydrolysate and standard sugar spots. Data in the table is the sum of average values for soluble and insoluble organic fractions. The six enamel samples contained an average of 0·4 per cent non-dialysable organic matter.

The approximate quantity of each aldose sugar recovered from the six pooled samples of teeth is shown in Table 1. An average of 1·65 mg total aldose sugar was recovered per 100 g adult enamel; 94 per cent was aldohexose, viz. galactose, glucose and mannose. The other three aldoses, tentatively identified as xylose, fucose and rhamnose, accounted for the remaining 6 per cent.

Hexuronic acid

All chromatograms developed in the pyridine solvent show a diffuse spot in the region of the glucuronic acid marker (Figs. 1 and 2). Interpretation is difficult due to the presence in the same area of slow-moving partial hydrolysis products. When the propionic acid solvent is used, hexuronic acids move farther than the partial hydrolysis products. Hydrolysate of insoluble enamel organic matter, chromatographed in

propionic acid solvent, displays a distinct spot with an R_f value similar to that of pure glucuronic acid (Fig. 3). Since glucuronic and galacturonic acids have similar R_f values in these solvent systems, the enamel hydrolysate spot could be due to either. The separation of galacturonic and glucuronic acids by means of Dowex 1 columns (KHYM and DOHERTY, 1952) is now being adapted to determine the type and amount of hexuronic acid present in adult enamel.

Hexosamine

The dark background of chromatograms stained for hexosamine by the Elson–Morgan reaction (PARTRIDGE and WESTALL, 1948) has made it impossible to produce clear-cut evidence that hexosamines are present in enamel organic matter. GARDELL (1953) has used ion exchange columns (Dowex 50) to separate galactosamine and glucosamine. This method is now being adapted to study the hexosamines in enamel organic fractions (NIKIFORUK, BURGESS and MACLAREN, 1959).

DISCUSSION

The low concentration of aldose sugars in enamel (1·65 mg%) has prevented crystallization and unequivocal identification of these sugars. However, the following evidence leads us to conclude that enamel contains three hexoses, two 6-deoxypentoses and one pentose. The enamel hydrolysate preparations, which were chromatographed, did not contain any positively charged molecules (e.g. amino-sugars or amino-acids) since these substances were adsorbed on the cation exchange resin. Each hydrolysate spot showed an R_f value identical to that of the same standard sugar when chromatographed in each of three different solvent systems. Each hydrolysate spot and its corresponding standard sugar showed the same colour reaction to the aniline hydrogen oxalate reagent—brown for hexose, orange brown for 6-deoxyhexose, and wine for pentose and deoxypentose. Approximately 94 per cent of enamel aldose was found to be hexose; made up of galactose, glucose and mannose. STACK (1954) had previously observed that enamel organic matter gives a positive reaction for hexose. The three hexoses that we have identified in enamel are also found as constituents of other animal tissues (GLEGG *et al.*, 1953; LEBLOND *et al.*, 1957). Chromatograms of enamel hydrolysates also displayed three faint sugar spots. Two showed a colour reaction with aniline hydrogen oxalate which is typical of deoxyhexose, and one showed the colour reaction of a pentose. One of the deoxyhexose spots had the same R_f value as fucose. Identification of this sugar is fairly certain since its R_f value in *n*-butanol–pyridine solvent is greater than those of the hexoses and less than those of all other deoxyhexoses (MACLENNAN, RANDALL and SMITH, 1959). The other deoxyhexose spot, tentatively identified as "rhamnose", could actually be one of five deoxyhexoses which have an R_f value close to that of rhamnose (MACLENNAN *et al.*, 1959). The pentose sugar from enamel hydrolysate had the same R_f value as xylose and was easily distinguished from arabinose or ribose in the *n*-butanol–pyridine solvent. KENT and WHITEHOUSE (1955) have stated that xylose can be formed by decarboxylation of glucuronic acid when chondroitin sulphate is subjected to acid hydrolysis. We have been unable to detect xylose when pure chondroitin sulphate was hydrolysed by cation exchange resin; nor do these

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hydrolytic conditions cause the formation of xylose from solutions of the standard sugars used for chromatographic markers. It is interesting to note that EASTOE and EASTOE (1954) recovered traces of xylose from acid hydrolysate of ox femur osseomucoid. Similarly, KUMAMOTO (1955) has identified xylose in dentine hydrolysed by cation exchange resin. Thus, there are indications that xylose is present in hydrolysates of organic matter from three calcified tissues.

The aldose sugars of enamel are not present as free monosaccharides but as constituents of molecules too large to pass through dialysis membranes. Prolonged hydrolysis is required to free them from their parent substance. The insoluble enamel organic matter contains over 80 per cent protein (STACK, 1955) which is associated with the aldose constituents. In the soluble organic fraction, too, the aldose seems to be part of a carbohydrate–protein complex, for STACK (1954) showed that the carbohydrate and protein moieties cannot be separated by electrophoresis. In many other tissues, the intensity of PAS staining is related to the concentration of protein-bound aldose sugars (LEBLOND *et al.*, 1957). The presence of such carbohydrate–protein complex in enamel could thus explain the PAS staining of enamel matrix noted by WISLOCKI and SOGNNAES (1950).

Carbohydrate–protein complexes rich in aldose have been isolated from mucous secretions (WERNER, 1953), non-mucous glandular secretions (LEBLOND *et al.*, 1957) and from many connective tissue derivatives (GLEGG *et al.*, 1954; KUMAMOTO, 1955; MEYER *et al.*, 1956; LEBLOND *et al.*, 1957). Such complexes from connective tissue derivatives invariably contain galactose, mannose and fucose, and have a hexose: methylpentose ratio greater than 9 (LEBLOND *et al.*, 1957). Enamel organic matter is typical of the group since it has these three aldose components and a hexose: methylpentose ratio of approximately 15. However, enamel differs from the other calcified members of the group, e.g. bone and dentine, in that glucose accounts for more than 25 per cent of the total aldose content. In this respect it is more akin to basement membrane and reticular fibres (GLEGG *et al.*, 1953).

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FIGS. 1–3. Paper chromatograms of sugars recovered from enamel organic matter.

Column (Exp). Sugars present in hydrolysates of either insoluble or soluble organic fractions from 7·5 g enamel.

Column (S1). Standard sugar mixture containing 1 γ/λ of galactose (ga); glucose (gl); mannose (ma); fucose (fu) and glucuronic acid (gl–a) most of which is converted to glucuronic acid lactone (gl–l) by the alkaline-pyridine solvent.

Column (S2). Standard sugar mixture containing 1 γ/λ of arabinose (ar); xylose (xy); ribose (ri) and rhamnose (rh). The chromatograms were developed in pyridine: *n*-butanol solvent unless otherwise noted.

FIG. 1a. Bovine enamel—hydrolysate of insoluble organic fraction.

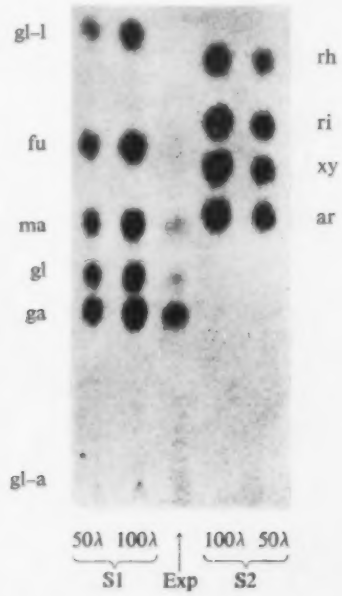

FIG. 1b. Bovine enamel—hydrolysate of soluble organic fraction.

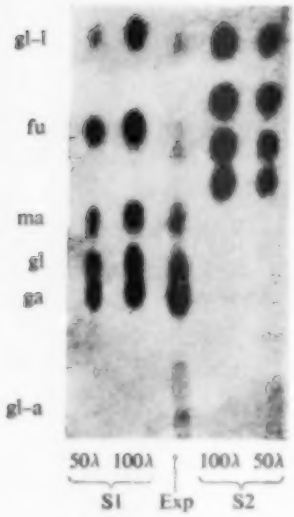

FIG. 2a. Human enamel—hydrolysate of insoluble organic fraction.

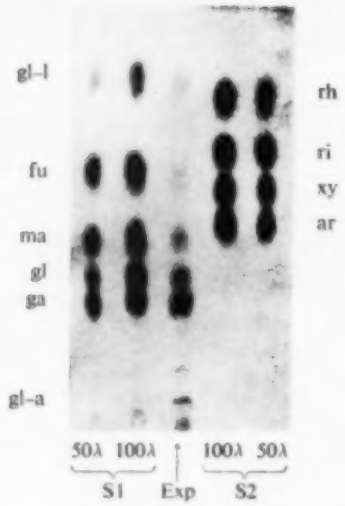

FIG. 2b. Human enamel—hydrolysate of soluble organic fraction.

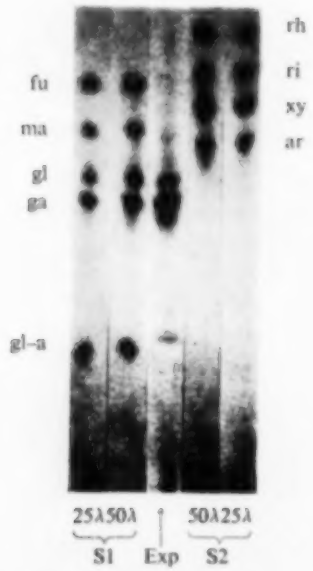

FIG. 3. Human enamel—hydrolysate of insoluble organic fraction chromatographed in propionic acid, propanol solvent.

PLATE 2

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EVALUATION OF THE DEGREE OF CARIES-SUSCEPTIBILITY IN STRAINS OF RATS

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Abstract—Two experiments were conducted to determine the influence on dental caries activity of maintaining rats of the Harvard caries-susceptible and caries-resistant strains together for various periods. In the first half of the first experiment, weanling rats of the two strains were caged together, while in the second half, weanling rats of the caries-resistant strain were housed with young adult rats of the caries-susceptible strain which had already developed numerous grossly detectable carious lesions. In the second experiment, half-litters were transferred from caries-susceptible mothers to caries-resistant mothers within 24 hr after birth. These foster offspring were maintained with the half-litter belonging to the mother until weaning at 21 days of age.

The rats of the caries-susceptible strain developed high rates of caries activity typical of their predecessors, regardless of the amount of contact with animals of the caries-resistant strain, or the lack of contact with members of their own strain. Three-quarters of the members of the caries-resistant strain which were maintained in direct contact with caries-active members of the caries-susceptible strain remained caries-free throughout the 250 day experimental period. The remaining quarter developed small numbers of slowly progressing carious lesions at a caries-activity level of approximately 10 per cent of that of caries-susceptible rats maintained on experiment for 80 days. The rats of the caries-resistant strain which became caries-active were restricted to a portion of the litters used in the experiments and probably represented the least resistant portion of the caries-resistant strain.

These data justify the concept that the caries-susceptibility and resistance of these strains are attributable to genetically inherited characteristics and that the terms caries-susceptible and caries-resistant can be correctly applied to the representatives of the two strains.

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INTRODUCTION

THE easy availability of weanling rodents with a predictable susceptibility to dental caries has been a frequent objective among dental investigators. Notable success in the long-term development through genetic selection of both caries-resistant and caries-susceptible strains of laboratory rats has been achieved at Michigan State University by HUNT, HOPPERT and ROSEN (1955) and at the Harvard School of Dental Medicine by WILLETT, RESNICK and SHAW (1958). Numerous other laboratories have developed and maintained their own caries-susceptible strains but have not sought to develop strains of caries-resistant animals. Similar attempts by JOHANSEN and HODGE (1956) over shorter periods of time have led to trends toward caries-resistance and caries-susceptibility among Syrian hamsters.

The caries activity of any strain of rodents tends to remain highly predictable when standard environmental conditions are maintained in the home laboratory. However, frequent examples of sharp changes in caries activity, most of which are undocumented and unpublished, have occurred where representatives of the strain

are transported to a new animal house in the same institution or when transferred to another city or country to establish a daughter colony. Many of these instances suggested that a strain of rats with the genetic potential for high caries-susceptibility could remain caries-inactive unless in contact with caries-active animals or housed in rooms where heavy concentrations of caries-active animals had been maintained.

The transmissibility of the essential caries-producing micro-organisms from caries-active animals has been demonstrated in a variety of circumstances by KEYES (1958, 1959, 1960) and FITZGERALD and KEYES (1960). These techniques not only demonstrated the concept of transmissibility with respect to experimental dental caries, but also provided procedures whereby further information could be garnered about the relative caries-susceptibility and caries-resistance of genetically selected strains of rats. The present studies were designed to provide varying degrees of contact between representatives of the Harvard caries-susceptible and caries-resistant strains of rats at varying periods in their life history to determine if they could influence each other's rate of caries activity and if they could justifiably be termed caries-susceptible and caries-resistant from a genetic standpoint.

EXPERIMENTAL PROCEDURE

The rats used as experimental subjects in these two experiments were representatives of two Harvard strains that have been maintained for approximately 10 years in these laboratories on our purified ration 2700 (GRIFFITHS and SHAW, 1960). The first strain was developed by genetic selection in each generation to emphasize susceptibility to carious lesions when the weanlings are fed diet 2700 or a comparable high carbohydrate ration. The representatives of this strain will be referred to throughout this paper as caries-susceptible rats. When maintained on diet 2700 after weaning, more than 80 per cent of the rats in the current generations have one or more grossly visible carious lesions by the eightieth day of age, the fifty-ninth day post-weaning; almost all the remaining rats have grossly visible lesions by the hundredth day of age, although the occasional rat did not have gross lesions until later.

The second strain has been developed to emphasize resistance to carious lesions when the weanlings are fed the same high carbohydrate diet 2700 throughout life. The representatives of the second strain will be referred to as caries-resistant rats in this paper. When maintained on diet 2700 after weaning, approximately 80 per cent of the caries-resistant rats in recent generations have remained caries-free throughout their normal lives; a low incidence of carious lesions has been observed in 20 per cent of these rats after maintenance on diet 2700 for 200 days or more. Gross lesions have not been observed prior to that age in representatives of recent generations.

Apart from the planned difference in genetic background, every effort has been made to provide identical environments for the breeding stock and experimental offspring from both strains. In addition to the continual use of ration 2700 for all breeding animals in both strains, they are housed in the same air-conditioned animal room, in identical cages with the same type of feeding dishes, bedding material, water bottles and water source. They are fed from the same ration container by the same technician on the same time schedule. When water bottles are refilled as needed, they

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are not sterilized and no attempt is made to ensure that any bottle is returned to its original cage, or indeed to a cage of the same strain from which it came. Cages are used interchangeably for both strains with washing in hot water and detergent but no sterilization between usage for different strains. These procedures were calculated to provide the maximum opportunity for similarity in diets, feeding and housing procedures, temperature and humidity, access to faeces and bacterial contamination.

Breeding policies have not been designed with the sole object of approaching homogeneity although decreased variability in dental caries incidence was desired. All potential breeding animals are carefully inspected for general evidence of health and vigour and, in addition, are given careful oral examinations at appropriate time intervals under a dissecting microscope in good light. In alternate generations, brother–sister matings are made with the most desirable male and female with respect to general physique and dental caries activity being selected from a litter. In the remaining generations, a desirable male with respect to the same characteristics is taken to be outbred with a female of desirable characteristics from another family line within the same strain. Due attention is paid to the family background of all selected rats to emphasize their resistance to intercurrent infections and to tumours, as well as their high productivity of vigorous offspring. The only difference in selection characteristics between the two strains has been the premium placed upon the early initiation of a high number of rapidly progressing carious lesions in the rats selected for breeding stock in the caries-susceptible strain and the search for the least incidence of carious lesions among the rats selected to produce the next generation in the caries-resistant strain.

The first experiment was designed to test the influence of allowing the weanlings of the caries-susceptible and caries-resistant strains to live together in pairs of the same sex in screen-bottom cages throughout a normal caries assay period. The design in this experiment is shown in the first five columns of Table 1. The first half of this experiment consisted of five groups of rats that were begun at weaning and maintained on caries-producing ration 2700 throughout. The rats in Group 1 were representatives of the caries-susceptible strain and were housed one to a cage. These rats compare to the usual caries-susceptible controls in other experiments reported from this laboratory. The rats in Group 2 were also caries-susceptible but were maintained in pairs of the same sex in screen-bottom cages of the same size. These rats served to test whether housing two rats in the same cage had any tendency to alter the dental caries experience under otherwise identical circumstances. In Group 3, pairs composed of a caries-susceptible and a caries-resistant rat of the same sex were maintained in screen-bottom cages. Group 4 was made up of pairs of caries-resistant rats of the same sex housed together. Group 5 consisted of caries-resistant rats housed singly. The latter group would compare to the usual caries-resistant controls in other experiments from this laboratory.

In addition to the daily feeding, the food cups of all rats in this experiment were checked a second time and refilled as needed. This precaution was taken to ensure that none of the paired rats was deprived of food lest any accidentally imposed caloric restriction reduce the dental caries experience. The caries-susceptible rats were

TABLE 1. THE INFLUENCE OF CAGING HARVARD CARIES-SUSCEPTIBLE AND CARIES-RESISTANT RATS SINGLY AND IN PAIRS ON THEIR CARIES-ACTIVITY

Group No.	Strain	Caging	Exp. period (days)	No. of rats	No. of caries-free rats	Gain in body weight (g)		No. of carious molars			No. of carious lesions			Extent of carious lesions		
						♂	♀	Avg.	SEM*	CR†	Avg.	SEM	CR	Avg.	SEM	CR
1	Susc.	Single	80	18	0	262	158	4·8	0·6	1·8	7·9	1·3	1·6	24·4+	4·3+	1·7
2	Susc.	Pair	80	20	0	250	147	6·2	0·5	1·0	10·7	1·3	0·7	35·3+	4·8+	0·7
3A	Susc.	Pair	80	18	1	263	141	5·7	0·7	3·9	9·2	1·3	3·4	24·8+	4·2+	6·8
A	Res.			18	18	270	168	0	0		0	0		0	0	
3B	Susc.	Pair	250	20	0	358	196	7·5	0·4		14·4	1·4		74·6+	6·1+	
B	Res.			20	16	419	264	0·7	0·3	0·8	0·7	0·3	0·8	1·4+	0·7+	2·0
4	Res.	Pair	250	18	17	395	242	0·1	0·1	1·0	0·1	0·1	1·0	0·1+	0·1+	1·0
5	Res.	Single	250	19	19	377	260	0	0		0	0		0	0	
6	Res.	Single	250	17	14	420	240	0·5	0·3	0·8	0·5	0·3	0·8	0·5+	0·3+	1·8
7	Susc.	Pair	250	34	0	—	—	7·4	0·4		16·2	1·0		68·8+	4·7+	
	Res.			34	26	377	265	0·8	0·2	0·8	0·8	0·2	0·9	1·7+	0·6+	0·5
8	Susc.	Single	250	16	0	—	—	6·8	0·6		14·8	1·2		64·9+	5·7+	

* SEM—Standard error of the mean.

† CR—Critical ratio. The critical ratio is the ratio of the difference between two means to the standard error of the difference between the means. Wherever the critical ratio is less than 2·0, the difference between the means is considered to be statistically insignificant; when from 2·0 to 2·9, moderately significant; when 3·0 or higher, highly significant.

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uniformly distributed among Groups 1, 2 and 3 with respect to litter and sex as were the caries-resistants in Groups 3, 4 and 5.

One-half (A) of the caries-susceptible rats in Group 3 and their caries-resistant cage mates as well as the caries-susceptible rats composing Groups 1 and 2 were killed after 80 days on experiment. Oral examinations *in vivo* at this age indicated that this experimental period was optimal for evaluation of the dental caries experience of the caries-susceptibles. The remaining pairs (B) in Group 3 and the caries-resistant rats of Groups 4 and 5 were killed after 250 days on experiment.

The second part of the first experiment was conducted to test whether maintenance of caries-resistant weanling rats for a full experimental period with young adult rats of the caries-susceptible strain that already had fully developed gross carious lesions would alter their expected resistance to experimental caries. This part of the experiment was composed of three groups of rats which were maintained on ration 2700. Group 6 consisted of caries-resistant weanlings maintained one to a screen-bottom cage. Group 7 was composed of a weanling caries-resistant rat in the same cage as a young adult caries-susceptible rat of the same sex that was observed to have a high incidence of rapidly progressing carious lesions. The caries-resistant rats in Groups 6 and 7 were distributed uniformly by litter and sex. Group 8 was composed of young mature rats of the caries-susceptible strain whose littermates were in Group 7. At the beginning of the experiment, the incidence of grossly visible carious lesions among the caries-susceptible rats of Groups 7 and 8 as determined by oral examinations *in vivo* was closely similar. All rats in the three groups were killed after 250 days on experiment. Again food dishes were checked twice daily in order that the larger rat did not consume all the available food at the expense of the smaller.

As a more stringent test, the second experiment was planned to test whether the nursing of caries-susceptible offspring from birth to weaning by females of the caries-resistant strain and caries-resistant offspring by females of the caries-susceptible strain would alter the expected dental caries experience. The design of this experiment is presented in Table 2. This experiment was composed of eight groups of offspring that were fed ration 2700 throughout the experimental trials. The first four groups were used to test the influence of the fostering procedure within strains, to determine whether this disturbance in the usual routine would alter the viability, the rate of growth or the inherently expected caries experience in either strain. Groups 5–8 compared the influence of the fostering procedure between the two strains.

The breeding females of both strains selected to produce offspring for this experiment were mated in large groups in order to have as many litters as possible born within 24 hr of each other for the fostering procedure. One-half of the babies in every suitable litter was left with the natural mother. In the first half of the experiment, the other half of the babies was exchanged with half of the offspring from another suitable female of the same strain. In the second half of the experiment, an identical exchange was made except that the half-litters were transferred to female rats of the opposite strain.

As nearly as possible one-half of the male and one-half of the female offspring were transferred in each case. For identification purposes, the fostered offspring were

Table 2. The influence of fostering rats by mothers of the same or different strain on the dental caries experience of the offspring

Group No.	Strain of offspring	Mother	Strain of mother	Exp. period (days)	No. of rats	No. of caries-free rats	Avg. weight of weanlings (g)		Avg. in body weight (g)		No. of carious molars			No. of carious lesions			Extent of carious lesions		
							♂	♀	♂	♀	Avg.	SEM*	CR†	Avg.	SEM	CR	Avg.	SEM	CR
1	Res.	Nat.	Res.	250	22	22	45	47	431	242	0	0	1·5	0	0	1·5	0	0	1·5
2	Res.	Fos.	Res.	250	27	24	49	46	390	230	0·3	0·2		0·3	0·2		0·9+	0·6+	
3	Susc.	Nat.	Susc.	80	34	0	51	47	236	145	5·6	0·3	0·1	9·2	0·7	0·6	27·3+	2·4+	0·6
4	Susc.	Fos.	Susc.	80	40	0	51	44	237	136	5·3	0·3		8·6	0·7		25·1+	2·3+	
5	Res.	Nat.	Res.	250	45	36	46	45	398	233	0·6	0·2	0·0	0·7	0·2	0·0	1·5+	0·6+	0·0
6	Res.	Fos.	Susc.	250	52	39	39	36	379	224	0·6	0·2		0·7	0·2		1·5+	0·6+	
7	Susc.	Nat.	Susc.	80	51	0	42	39	255	109	5·5	0·3	1·2	9·7	0·8	0·8	28·7+	2·7+	0·7
8	Susc.	Fos.	Res.	80	47	1	44	43	214	115	6·1	0·4		10·6	0·9		31·6+	3·0+	

* SEM—Standard error of the mean.
† CR—Critical ratio.

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marked by rubbing a little India ink into a tiny cut in the left ear. This tattoo had to be renewed at 5–6 days of age due to the rapid growth of the ear, after which the mark was clearly discernible until long after weaning.

Thus the rats in Groups 1 and 2 were littermates of the caries-resistant strain; the ones in Group 1 were nursed by their own mothers while their brothers and sisters in Group 2 were nursed by foster caries-resistant females. The rats in Groups 3 and 4 were littermates of the caries-susceptible strain; the representatives of each litter in Group 3 were nursed by their own mothers while their siblings in Group 4 were nursed by foster caries-susceptible females. The rats in Groups 5 and 6 were littermates of the caries-resistant strain; those rats in Group 5 were suckled by their natural mothers while their brothers and sisters in Group 6 were nursed by foster caries-susceptible females. The foster mothers used in Group 6 were the true mothers of the caries-susceptible rats in Groups 7 and 8. The subjects in Groups 7 and 8 were littermates of the caries-susceptible strain; the offspring in Group 7 were reared by their own mothers while their brothers and sisters in Group 8 were nursed by foster caries-resistant females. The foster mothers in Group 8 were the mothers of the caries-resistant experimental subjects in Groups 5 and 6.

The rats in Groups 1, 2, 5 and 6 whose ancestry was the caries-resistant strain were continued on experiment for 250 days while the rats in Groups 3, 4, 7 and 8 which were genetically of the caries-susceptible strain were killed after 80 days on experiment.

At the termination of the appropriate experimental period, the rats were sacrificed under ether anaesthesia and the heads fixed in 95% ethanol. After at least 48 hr of fixation, the heads were skinned and evaluated for the number of carious molars, the number of carious lesions and the extent of carious lesions by the procedure developed by SHAW *et al.* (1944).

RESULTS

The results of the first experiment are presented in Table 1. In the first half of the experiment where all five groups were composed of weanlings from the two strains caged in different fashions, no important differences in the rate of increase in body weight were observed among the male or female rats of either strain. The average dental caries incidences for the caries-susceptible rats in Groups 1, 2 and 3A did not differ significantly from each other despite the three caging arrangements. The average dental caries indices for the caries-susceptible rats in Group 3B were significantly higher than for those of the previous three groups because of the additional 170 days on experiment. Not one of the eighteen caries-resistant rats housed with caries-susceptible rats for 80 days in Group 3A had any evidence of dental caries. In this regard, the caries-resistant rats had behaved true to genetic form, as freedom from caries at this age has been universally true in the last twenty or more generations of this strain. Four of the twenty caries-resistant rats housed with caries-susceptible rats for 250 days in Group 3B developed a small number of relatively small, slowly progressing carious lesions. Only one of the eighteen caries-resistant rats living in pairs in Group 4 and none of the nineteen living singly in Group 5 developed carious lesions. The low level of dental caries incidence among the caries-resistant rats in

Group 3B is not significantly higher than for the rats in Groups 4 and 5 except in the category of the extent of carious lesions when the difference is significant at the 5 per cent level.

The average caries activity among the four caries-active rats in Group 3B was less than 10 per cent of the average caries activity of the caries-susceptible rats with which they were housed. This low level of caries initiation and progression suggests that these four rats had inherited a somewhat lower resistance to caries than most representatives of the strain, so that the transmission of the caries-producing oral flora from caries-active rats was capable of initiating a minor caries activity. The remaining sixteen rats in the group had the same opportunities for transmission but remained caries-free.

The dental caries incidences of the caries-susceptible rats in Groups 7 and 8 were very closely similar after the 250 day experimental period. Of the seventeen caries-resistant rats in Group 6, fourteen remained caries-free throughout the experimental period, as did twenty-six of the thirty-four in Group 7. Again in this half of the experiment, the caries-resistant rats that were housed with caries-susceptible rats tended to have a slightly elevated incidence of small, slowly progressing carious lesions. Unlike the first half of the experiment, the difference in the category of extent of carious lesions was not of borderline statistical significance. Again the eight animals of the caries-resistant strain that developed carious lesions had a minor caries activity even after this prolonged period that was slightly less than 10 per cent of the caries activity of the caries-susceptible rats with which they were housed.

In both halves of this experiment there is a slight trend toward an increased caries activity among caries-resistant rats housed with caries-susceptible rats throughout a complete experimental period. This observation suggests that a small percentage of the resistant strain is just barely susceptible enough to develop a minor caries activity under circumstances where transmission of caries-producing oral micro-organisms is made possible. However, in both halves of the experiment 75–80 per cent of the representatives of the caries-resistant strain were sufficiently caries-resistant that this impact was insufficient to cause the development of any carious lesions.

In both halves of the first experiment there is no suggestion that the caries incidence of the groups of caries-susceptible rats housed for prolonged periods with caries-resistant rats has been altered in any way.

The results of the second experiment are presented in Table 2. Throughout Groups 2, 4, 6 and 8 where the offspring were fostered, they grew as well during the lactation period as their littermates in Groups 1, 3, 5 and 7 which were cared for by their own mothers. No difference was observed between the mortality rates of the offspring, whether nursed by their own or by foster mothers. The average gains in body weight during the respective post-weaning experimental periods did not differ significantly between natural and fostered offspring in either strain.

The average dental caries incidences for Groups 1, 2, 5 and 6 of caries-resistant rats did not differ significantly from each other. None of the twenty-two caries-resistant rats suckled by their natural mothers became caries-active; three of twenty-seven caries-resistant rats fostered by other caries-resistant females had low incidences of dental caries. Likewise, nine of forty-five caries-resistant offspring nursed by their

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own mothers and thirteen of fifty-two caries-resistant rats fostered by caries-susceptible females had low incidences of dental decay. However, in each case the amount of tooth decay was extremely low compared to the amount of tooth decay experienced by caries-susceptible offspring in one-third of the experimental time. It seems highly improbable that the oral cavities of the thirty-nine offspring of the caries-resistant strain that did not become caries-active did not have ample opportunity to become repeatedly inoculated with the same flora that became established in the mouths of their foster littermates.

The average dental caries incidences for the caries-susceptible rats in Groups 3, 4, 7 and 8 were not significantly different from each other irrespective of whether they were nursed by their own mothers, by other caries-susceptible females or by caries-resistant females. Of the 172 caries-susceptible rats in these four groups, only one fostered by a caries-resistant mother failed to develop active carious lesions in the 80 day experimental period.

The caries-susceptible rats in Group 8 that were fostered by females of the caries-resistant strain developed an incidence of dental caries that was entirely comparable to that of representatives of the strain during recent generations. Their contact with their natural mothers lasted for 24 hr or less after birth. Hence if their oral flora was established as a result of contact with their mothers, it must have occurred during this brief period and been maintained throughout the nursing period and for the 15–18 days prior to the eruption of molar teeth. Our experimental conditions do not rule out this possibility. If this inoculation did not occur or if it was inadequate, the caries-susceptible offspring must have received the appropriate oral flora from other sources.

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DISCUSSION

Unexpected changes in caries activity among laboratory animals have been observed with considerable frequency under conditions where the change could not be attibutable to an hereditary origin. The hamster has been frequently involved in such incidents with complete or almost complete absence of caries in strains that for generations had demonstrated an excellent level of caries activity. Most incidents have been undocumented except by the temporary or permanent cessation of publications from various laboratories where active investigation with the hamster has been conducted. However, KEYES (1954) has reported one experience when hamsters were moved from the caries-susceptible populations at the University of Rochester to the Harvard School of Dental Medicine. Despite the use of the most cariogenic diets known, all offspring of this daughter colony remained essentially caries-free throughout several generations.

Comparable incidents with the laboratory rat have been less critical and have received less attention, but may well have occurred as frequently. Our caries-susceptible strains were maintained during the early generations of the selective breeding programme in rented quarters in the Harvard Medical School. With the completion of a new animal laboratory in the Harvard School of Dental Medicine, the entire population of several hundred rats was moved in the course of a few days. The animals in the next two or three generations demonstrated a great deal lower caries

activity than we had observed during the previous several generations. Dietary procedures and methods of genetic selection had not been changed in this interval. Since no rats of the same strains had been continued in the old quarters, no strict controls were available; however, the change in caries activity appeared to be attributable to the environmental change to an area where no rats with carious lesions had been maintained previously.

In time, desirable levels of caries activity were regained so that suitable offspring were available from the colony for experimental purposes here as well as for shipment of breeders to establish daughter colonies elsewhere. On at least two occasions, the one when rats were shipped to the University of Liverpool, England, and the other when rats were shipped to the University of Mainz, Germany, the offspring of this stock maintained under fairly comparable conditions to those in our laboratories had low rates of caries activity. In both cases, brothers and sisters of the rats shipped were maintained in our colony and produced offspring with a comparable caries activity to that of the entire caries-susceptible strain.

A reverse example occurred in our laboratories when representatives of the Hunt–Hoppert caries-susceptible and caries-resistant strains were shipped to us in 1955. The caries-susceptible strain whose adult members had many gross carious lesions upon arrival produced offspring which gave every evidence of being very susceptible under our laboratory conditions and have continued to do so. However, the adult members of the caries-resistant strain which had no gross carious lesions upon arrival and developed none during their normal lives bore offspring which under our experimental conditions proved to have a high caries activity on the average, with a great variation from rat to rat. When the more resistant rats were selected and inbred during the next three generations, the offspring in the succeeding generations rapidly began to demonstrate a high caries-resistance on the same dietary regimens. The ease with which a consistent and high degree of caries inactivity was attained was notably unlike the situation encountered in the development of a caries-resistant strain from a completely mixed population.

The data from the two experiments described in this paper provide support for our hypothesis that the representatives of our caries-susceptible and caries-resistant strains have inherited these characteristics by truly genetic pathways and do not become caries-active or caries-inactive because of the availability or unavailability, respectively, of the appropriate microbiota. Offspring of the caries-susceptible strain when maintained without contact with caries-active rats or their excreta from within 24 hr after birth for the rest of their experimental lives developed carious lesions at the expected rate. A suitable caries-producing flora evidently was as readily established in these rats that were maintained out of contact with caries-active animals as their littermates which had the maximum contact with caries-active animals. The source of this oral flora is unknown at present. Possibly the brief contact with the caries-active mother after birth provided a sufficient inoculum to permit establishment of the flora even before molar teeth had erupted. If this did not occur, the micro-organisms may have been available from the air as these experiments were conducted in rooms where large concentrations of caries-active rats were maintained on other experiments.

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Other possibilities are transmission from the hands of the technicians who did the feeding, watering, weighing, etc., or from the caries-resistant foster mother who may harbour the caries-producing flora without being influenced by their presence.

Forty-two out of fifty-four offspring of the caries-resistant strain maintained after weaning throughout the 250 day experimental period with weanlings of the caries-susceptible strain or with young caries-active adults of the caries-susceptible strain were not rendered caries-active by this amount of contact. In addition, thirty-nine of the fifty-two caries-resistant offspring suckled by caries-susceptible mothers mixed in litters with caries-susceptible offspring remained caries-inactive. It seems to be inconceivable in any one of these three experimental circumstances that ample opportunity for the transfer of caries-producing organisms to the oral cavities of the caries-resistant rats did not occur frequently. However, in the midst of these opportunities for contact, the great majority of the rats from caries-resistant parentage were able to remain free not only of grossly visible carious lesions, but even of minute lesions in the enamel.

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Twelve of the caries-resistant rats in Groups 3B and 7 of Experiment 1 did become caries-active at a low level. In contrast, only four out of fifty-four caries-resistant rats in Groups 4, 5 and 6 became caries-active when maintained without contact with caries-susceptible rats. Tracing back through the records of parentage, the sixteen rats that became caries-active in the first experiment were from six of the sixteen litters represented in all groups and were the offspring of six females and four males. These ten breeding animals were from four litters: two females were from one litter, one male and one female were from a second litter, both of these litters had the same father, another two males and two females were from a third litter and the last male and female were from a fourth litter, the members of which were first cousins of the animals in the third litter. Thus the rats from the caries-resistant strain in Experiment 1 that developed carious lesions were closely related to each other.

A similar situation pertains in Experiment 2. Only twenty-five of the 146 caries-resistant offspring demonstrated even a minimal level of caries activity. These caries-active rats were from ten of the twenty-one caries-resistant litters used and were the offspring of nine females and five males. One litter alone contributed eleven of the twenty-five caries-active subjects. As in Experiment 1, the parents were from a small group of litters in a closely related line of the caries-resistant strain.

It seems reasonable to assume that the caries-active rats in Experiments 1 and 2 represented a somewhat less resistant segment within the overall population of the caries-resistant strain. Some of these caries-active rats were not in close contact with caries-susceptible rats and yet developed a small number of minor carious lesions. The majority of the caries-active rats had been in contact with caries-susceptible rats at some period of their lives and presumably had had the opportunity to receive an appropriate inoculation of the caries-producing flora from their caries-active cage-mates. Their somewhat lower caries-resistance than the other representatives of the caries-resistant strain that had the same potential amount of contact with caries-active rats was inadequate to enable them to maintain intact teeth throughout the experimental period.

In each group of caries-resistant rats in Experiments 1 and 2 that were allowed any contact with caries-active rats from the caries-susceptible strain at different periods in their life history, an appreciably higher number became caries-active than in the groups of caries-resistant littermates maintained without contact with caries-active rats. Although the caries activity of these rats from the caries-resistant strain was always at a minimal level, these increases in numbers of caries-active subjects give support to the Keyes' hypothesis of transmissibility. Indeed, there is a likelihood that the experimental provision of an increased amount of contact between caries-active rats and representatives of the caries-resistant strain that are potential breeders may serve as an additional way to select caries-resistant rats for the breeding stock in each new generation.

However, the data from Experiments 1 and 2 are of greater importance than the support that they give to Keyes' concept of transmissibility. First, these data clearly indicate the predictability with which representatives of the Harvard caries-susceptible strain of rats in our laboratory environment develop high rates of caries activity even when maintained without direct contact with caries-active rats. Secondly, the data indicate the high degree of caries-resistance within the Harvard caries-resistant strain. Despite the opportunity for transfer of appropriate flora from caries-active rats, three-quarters of the representatives of this strain remained caries-free in an experimental period three times the length of the period for the caries-susceptible strain; the remaining quarter of the representatives became caries-active at a level averaging 10 per cent of that of the caries-susceptible rats in the shorter period. On the basis of present knowledge, these observations appear to justify the concept that the Harvard caries-susceptible and Harvard caries-resistant strains express these characteristics by reason of genetically inherited influences. Thus the strain designations "caries-susceptible" and "caries-resistant" can be used advisedly to connote constitutionally determined traits.

Other experimental data also indicate the strength with which caries-susceptible and caries-resistant characteristics are determined genetically. HALL and SHAW (1958) parabiosed pairs composed of a caries-resistant and a caries-susceptible rat of the Harvard strains and maintained them on our customary cariogenic diet. Under the conditions of this experiment, the parabionts developed the same amount of tooth decay as their single littermates. In addition to the environmental opportunities for transfer of micro-organisms between strains, a slow intermingling of the blood of the two rats occurred. In an investigation that was similar to our second experiment, ROSEN, HOPPERT and HUNT (1960) transferred entire litters within 24 hr after birth from a caries-susceptible to a caries-resistant mother and vice versa. The subsequent litters born to these rats were used as controls. The preliminary results indicated that the fostered offspring have developed incidences of experimental dental caries that were typical of their genetic inheritance and unlike that of their foster mothers.

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RECENT ADVANCES IN THE INVESTIGATION OF THE CRYSTAL CHEMISTRY OF DENTAL ENAMEL

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Abstract—In view of detailed investigations carried out by the writer and several other individuals, the conclusion is inescapable that dental enamel is composed of a single "mineral" phase which is carbonate hydroxyapatite (dahllite). There is no valid reason for supposing that the crystal structure or crystal chemistry of bone or dentine is significantly different. Even in bovine bone no appreciable isotopic exchange with C^{14} was observed by FRANÇOIS (1958), so the concept of adsorption of carbonate on the surfaces of the crystallites can be relegated to unrealistic speculation. The writer's structural theory (1952) is essentially confirmed.

INTRODUCTION

IN 1952 attention was called to the relationship between the carbonate–apatite mineral, francolite, and the "mineral" phase of teeth and bones, and a fairly complete structural theory for the carbonate apatites was presented (MCCONNELL, 1952a). The major postulate of this theory has been accepted by mineralogists and now appears in the principal reference books by STRUNZ (1957), EITEL (1954) and PALACHE, BERMAN and FRONDEL (1951). This theory, to be sure, was established on the basis of a carbonate apatite containing appreciable amounts of fluoride (francolite) rather than the carbonate hydroxyapatite (dahllite), but recent results have not only permitted the extension of this theory to include dahllite (MCCONNELL, 1960a) but a hydrated calcium aluminum silicate (scawtite) as well (MCCONNELL and MURDOCK, 1958). The question concerning the presence of carbonate ions in apatite is not new, of course, but arose in 1822 (HAÜY).

DISCUSSION OF EXPERIMENTAL RESULTS

Recent investigations by several persons, however, have resolved this question beyond reasonable doubt with respect to the minerals dahllite and francolite. AMES (1959), for example, has demonstrated (a) that a calcium phosphate can be synthesized which contains as much as 10 per cent of carbonate, (b) that this substance has essentially the structure of apatite, and (c) that the carbonate is essentially within the apatite structure rather than located on the surface of the crystallites. The significance of AMES' contribution with respect to an understanding of teeth and bones can be appreciated only through the realization that it demonstrates the futility of attempts to learn anything about the structure of teeth and bones by such methods as dissolving them in acids or heating them.

STEEL (1960), for example, has recently made an elaborate study of the dissolution of dental enamel and found the Ca/P weight ratio in solution to range from 0·38 to 7·00 at pH 5·0. At lower pH values he found the Ca/P ratios to show narrower ranges, and at pH 2·0 he found a mean ratio of 2·30. From these data, in combination

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with several erroneous assumptions, he deduces that dental enamel contains a second phase, namely $CaCO_3$ in "amorphous form", because "$CaCO_3$ is more soluble in hydrochloric acid solution than hydroxyapatite". This experiment, of course, is comparable to one in which an attempt might be made to ascertain the metallic composition of an alloy by analysing the solution after *partial* dissolution has taken place. Obviously this could be done only after the comparative dissolution rates and their mutual influences upon each other were known with respect to each metal present and with respect to time, pH, formation of complex ions, etc. STEEL's results, although interesting, are non-critical with respect to any conclusions related to the composition of the solid phase of dental enamel.

From the viewpoint of fundamental crystal-chemical theory, the results of AMES (1959) are not surprising, but had been theoretically anticipated, at least in a qualitative way. However, some workers are still reluctant to admit the direct applicability of these, and numerous other, data and conclusions to teeth and bones. They are perplexed by large quantities of data on Ca/P ratios, so-called exchange reactions, etc., which have been published at various times in various places. Most of these data do not disprove the close analogy between dental enamel and dahllite; in fact, in view of the discrepancies which exist, it can fairly be said that they do not prove or disprove anything, inasmuch as many of the conclusions based thereon are *non sequiturs* (MCCONNELL, 1955a,b).

TABLE 1. RATIOS OF LARGE CATIONS TO PHOSPHORUS FOR CARBONATE-APATITE MINERALS
(By permission from *The American Mineralogist*)

Mineral	CO_3 (Wt.%)	Locality and reference*	Ca′/P′ (Atomic)	Ca′	P′
Dahllite	2·72	Bluffton, Ohio MCCONNELL, 1960	1·69	Ca, Mg, Na	P
Dahllite	4·46	Mouillac, France MCCONNELL, 1938	1·72	Ca, Na, K†	P, S
Dahllite	3·36	St. Paul's Rocks, Atlantic WASHINGTON, 1929	1·97	Ca	P, S
Francolite	5·45	François Lake, B.C. POITEVIN, 1927	1·82	Ca§	P
Francolite	1·80	Busumbu, Uganda DAVIS, 1947	1·77	Ca, Mg	P
Francolite	3·36	Staffel, Germany GRUNER and MCCONNELL, 1937	1·85	Ca, Mg	P, V
Francolite	4·43	Richtersveld, South Africa DE VILLIERS, 1942	2·02	Ca, Mg, Na	P
Francolite	1·98	Yorkshire, England DEANS, 1938	1·81	Ca, Sr	P
Francolite	3·40	Tavistock, Devon, England SANDELL *et al.*, 1939	1·80	Ca, Mg	P

* References for the Table are given by MCCONNELL (1960a), except WASHINGTON, H. S. (1929), *Amer. Min.* **14**, 369.

† Oxides disregarded as "impurities" were: Fe_2O_3, Al_2O_3, SiO_2.

§ Disregarded were: FeO, Fe_2O_3, Mn_2O_3, Al_2O_3, MgO, SiO_2 and organic C.

Of particular interest are Ca/P ratios of the solid material. Extensive analytical results on teeth and bones have appeared, as well as those on various synthetic substances which gave apatite-like diffraction patterns. With respect to bones, KLEMENT's (1938) results are of interest because all of his molar ratios, except for three avian samples, exceed the theoretical value for hydroxyapatite (1·667). Likewise all of the Ca/P ratios for mineral carbonate apatites exceed the theoretical value when magnesium and sodium equivalents are summed with calcium (MCCONNELL, 1960a). Indeed, this ratio was found to be as high as 2·02 for francolite from South Africa (Table 1). All of these data are consistent with the theory on the replacement of phosphorus by carbon in the apatite structure, but one cannot expect a direct correlation between carbonate content and the Ca/P ratio for reasons which will be discussed.

TABLE 2. STRUCTURAL PERIODICITIES OF HYDROXYAPATITE
(By permission from *Die Naturwissenschaften*)

Reference*	a_0(Å)	c_0(Å)
BALE	9·49 ± 0·03	6·89 ± 0·03
POSNER and STEPHENSON	9·45	6·89
JENSEN and MØLLER	9·450 ± 0·002	6·871 ± 0·001
THEWLIS, GLOCK and MURRAY	9·43	6·88
KLEMENT and DIHN	9·42	6·94
HENDRICKS *et al.*	9·42 ± 0·03	6·94 ± 0·03
POSNER, PERLOFF and DIORIO	$9{\cdot}43_2$	$6{\cdot}88_1$
WALLAEYS (precipitated)	9·410 ± 0·005	6·865 ± 0·002
WALLAEYS (hydrothermal)	9·403 ± 0·001	6·886 ± 0·001
TRAUTZ	9·421 ± 0·003	6·881 ± 0·003
CARLSTRÖM	9·421 ± 0·001	6·882 ± 0·001
PERDOK	9·420 ± 0·001	6·885 ± 0·001
BRASSEUR	$9{\cdot}41_9$	$6{\cdot}88_7$
BRASSEUR†	$9{\cdot}40_8$	$6{\cdot}86_9$
WALLAEYS§	9·44 ± 0·01	6·860 ± 0·005

* References for the table are given by MCCONNELL (1960b).
† So-called tricalcium phosphate dihydrate.
§ Aqueous precipitated tricalcium phosphate.

Synthetic "hydroxyapatite" (including so-called tricalcium phosphate hydrate) has been reported to show Ca/P from 1·95 to as low as 1·33, although it is usually admitted that more than one phase is present when the lower ratio is obtained (ARNOLD, 1950). Re-examination of X-ray diffraction data on various "hydroxyapatites" clearly indicates (Table 2) that these measurements could not have been made on the same pure crystalline phase because the discrepancies exceed the experimental errors by large factors (MCCONNELL, 1960b). Under these circumstances it becomes necessary to assume not only that the unit cell dimensions fail to indicate those of pure hydroxyapatite, but that the reported Ca/P ratios are either those of mixtures (containing more than one phase) or are those of variants containing

isomorphic combinations, such as:

$$Ca_{10}(OH)_2(PO_4)_6(CO_3OH) \text{ and/or}$$
$$Ca_{10}(H_2O_2)(PO_4)_4(O_4H_4)_2$$

which have Ca : P : H_2O equal to 2·00 : 1 : 0·30 and 2·50 : 1 : 1·50, respectively.

It is to be noted that although both of these hypothetical compounds have Ca/P ratios which exceed 1·67, only one of them contains carbon dioxide. Consequently no correlation between the Ca/P ratio and the carbon dioxide content would be expected, and none is observed.

In general, it can be assumed that Ca/P ratios less than the theoretical value are to be accounted for either (a) because of failure to include Na and Mg with Ca, or (b) because of the presence of other extraneous phases, such as brushite or octacalcium phosphate (BROWN *et al.*, 1957). The near coincidence of most of the more intense X-ray diffraction maxima of these substances with those of apatite could readily prevent detection of small amounts of these substances.

So-called alpha tricalcium phosphate hydrate not only fails to show consistent analyses with respect to the amount of water present, but it merely approximates Ca/P=1·5. Reports on the *a* periodicity of this precipitate are 9·44 ± 0·01, according to WALLAEYS (1952) and 9·40$_6$ Å according to BRASSEUR (1958). It should be obvious that the compositions of these two substances must be significantly different. Equally obvious is the futility of attempting to reconcile something with Ca/P=1·5 with the apatite structure, because (a) not only does this supposition require vacancy among one-fourth of the calcium positions of one symmetrical sort, but (b) it requires that these vacancies must be random, because their symmetrical location would surely give rise to recognizable structural differences which have not been observed. Indeed, inasmuch as this proposal involves omission of one calcium atom of the four-fold position (1/3, 2/3, 0), the planes of symmetry at 1/4 and 3/4, as well as the 6_3 axis and the two-fold axes, would necessarily become inoperative, and the space-group symmetry could no longer be that of hydroxyapatite ($P6_3/m$). An alternative is to assume that every fourth unit cell has all four calcium positions vacant, a proposition which becomes equally untenable. It is deduced, then, that such a substance as $Ca_3(PO_4)_2 \cdot 2H_2O$ cannot possibly be isostructural with hydroxyapatite on the basis of fundamental crystallographic considerations. Since all substances with this alleged composition give the diffraction pattern of hydroxyapatite, there is no valid evidence for the existence of any such compound as tricalcium phosphate hydrate.

In a recent attempt to obtain an explanation of the isostructural character of hydroxyapatite and tricalcium phosphate hydrate, BRASSEUR (1958) calls upon the results of MERKER and WONDRATSCHEK (1957) who prepared some lead-containing apatites with the supposed composition $Pb_4A(YO_4)_3$, where A = Na, K, Rb, Cs, Tl; and Y = P, As, V. On this basis BRASSEUR assumes that $Ca_4H(PO_4)_3$ also would be isostructural, but this assumption violates one of the principal rules of crystal chemistry: hydrogen, in general, will not isomorphously replace alkali ions, and vice versa. Thus his theoretical explanation—he gives no experimental evidence—is quite unacceptable.

It must be admitted that knowledge of the crystal chemistry of bone is considerably complicated by the extremely small size of the crystallites, but the inherent

natural difficulties have been made appreciably worse by misguided interpretations of non-critical experiments. However, the relationship between dental enamel and dahllite is a straightforward one which is not confused by the size of the crystallites. Thus we have, in terms of the X-ray diffraction data, most excellent correlation between human dental enamel and a fossil dental enamel of a mastodon (MCCONNELL, 1960a). Both of these substances produce very good, if not excellent, diffraction patterns (Figs. 1 and 2). Of course, both contain appreciable amounts of carbon dioxide but give no evidence whatever of consisting of more than one crystalline phase.

The suggestion has been made that an amorphous phase might be present, and might contain both carbonate and phosphate ions in addition to calcium. This suggestion surely deserves consideration in view of a vast accumulation of geochemical knowledge. There is no amorphous mineral substance known to occur in nature which contains any such quantity of carbon dioxide as occurs in bone. Furthermore, there is no naturally occurring amorphous mineral substance which consists essentially of calcium phosphate. With respect to bone, it can be concluded that the amount of carbon dioxide present probably could not be accounted for even if the entire substance were amorphous. Certainly in the case of dental enamel, which appears to be essentially crystalline by both optical and X-ray methods, the carbonate content cannot be present as a constituent of an amorphous phase.

The suggestion has been made that the carbonate is adsorbed on the surfaces of the crystallites of bone. Again, these conclusions were based on non-critical experiments, which are not subject to unique interpretation. Recent experiments using C^{14} as a tracer have indicated that no appreciable amount of the carbonate is subject to isotopic exchange. FRANÇOIS (1958) found this to be true for bovine bone, and AMES (1959) found it to be true of his synthetic carbonate apatite. AMES should be quoted in this connection; he states: ". . . this carbonate can be entirely present within the apatite lattice when the apatite contains less than 10 per cent by weight CO_3^{-2}". This amount, to be sure, is more than twice the quantity present in bone or dental enamel. AMES states further: "Structural formulae, equilibrium data and C^{14} tracing show no indications of the carbonate being present in Hendricks' 'voids' or adsorbed to any great extent on apatite surfaces . . ."

Another conclusion of AMES is interesting: "The variable composition of the apatite phase, even in this relatively simple system, shows the fallacy of attempting to apply the laws of sparingly soluble compounds to apatite". In 1952, at the Josiah Macy, Jr. Conference on Metabolic Interrelations, the "incongruent solubility of silicates" was referred to by me (MCCONNELL, 1952b, p. 171) in an attempt to point out that one would not expect bone or tooth substance to behave differently from most silicates. One versed in silicate chemistry surely would not expect to be able to write a "solubility product" for bone mineral. Although I may have introduced the phrase, "incongruent solubility" to emphasize the important similarities, nevertheless, such principles are well known to silicate chemists who surely know that feldspar can be dissolved in water or acid, but nothing remotely resembling feldspar will re-precipitate from this same solution at ordinary temperature within a reasonable length of time—perhaps 10 years.

The fallacy of depending solely upon X-ray diffraction data as a basis for deciding whether dental enamel, for example, is composed of hydroxyapatite or a carbonate hydroxyapatite was demonstrated by OSMOND and SAWIN (1959). They accurately measured forty samples of recent and fossil teeth of vertebrate animals. Some representative measurements of *a* periodicities were as follows (in Ångström units): 9·45 (human), 9·40 (Miocene rodent), 9·42 (recent lungfish), 9·37 (Permian lungfish), 9·39 (recent shark), 9·37 (Permian shark). They conclude "that fluoridization alone is not the cause of the change with time of vertebrate tooth apatite unit-cell dimensions". These findings and conclusions, including the smaller *a* dimension for a recent shark than for a Miocene rodent, are entirely consistent with the theory (MCCONNELL, 1960b) that the variations in carbon dioxide and hydroxyl contents influence these dimensions, as well as the fluorine content. Thus it is completely meaningless to state that human dental enamel is hydroxyapatite because unit cell dimensions closely resemble those of "hydroxyapatite". The question immediately arises: which "hydroxyapatite" in Table 2? The fundamental emphasis, to be sure, must be placed on the fact that all of these substances contain significant, although variable, amounts of carbon dioxide.

Such differences in structural periodicities as those observed by OSMOND and SAWIN (1959) cannot be explained on the basis of variations in the amount or composition of some admixed amorphous constituent. These crystal-chemical differences must be produced by compositional variations among these specimens.

CONCLUSIONS

Biochemists do not seem to be reluctant to call the carbonate hydroxyapatite of bone and teeth a "mineral" substance, and they refer to its formation as "mineralization"—in this sense meaning merely the formation of mineral matter, apparently. However, if they are quite willing to borrow the terms—and there are good reasons for their doing so—one might raise the important question: why are they reluctant to borrow the fundamental principles which apply to mineralogical chemistry also? At this juncture it might be mentioned that the term, mineralization, implies something beyond the mere formation of a mineral to most mineralogists, but this is a minor distinction over which we can pass without further consideration.

However, the complete disregard of applicable mineralogical and crystal-chemical concepts cannot be considered a trivial matter. The composition of teeth and bones cannot be correctly expressed as hydroxyapatite. These substances are carbonate hydroxyapatites or, if one wants a good mineralogical term, dahllite.

We have made no attempt to discuss the details of several new lines of evidence which indicate the carbonate-apatite composition of teeth and bones. My own results have been integrated with those of AMES (1959), FRANÇOIS (1958) and MASLENNIKOV and KAVITSKAYA (1956). These results were concerned with fossil dental enamel, synthesis of carbonate apatite, bovine bone, and the phosphorite minerals. All of these data point toward the principle that the carbonate component is within the lattice of the apatite structure, and, furthermore, that no significant amount of carbonate is adsorbed on the surface of the crystallites. All of these investigations,

except the work of FRANÇOIS, were concerned directly with the question of the isomorphic substitution of carbon for phosphorus, and in all cases this fundamental premise was verified—completely vindicating those mineralogical authorities who had already accepted this theoretical concept on the basis of earlier evidences.

The textbooks which refer to the substance of dental enamel and bone as "hydroxyapatite" contain a fundamental error, which now has been repeated so often, and in so many places, that it will probably require many years to correct this error. Twenty-three years have already elapsed since GRUNER, MCCONNELL and ARMSTRONG (1937) pointed out that this substance is a carbonate hydroxyapatite. BOGERT and HASTINGS (1931) had done so earlier, but could not reconcile their formula with the crystal structure of apatite.

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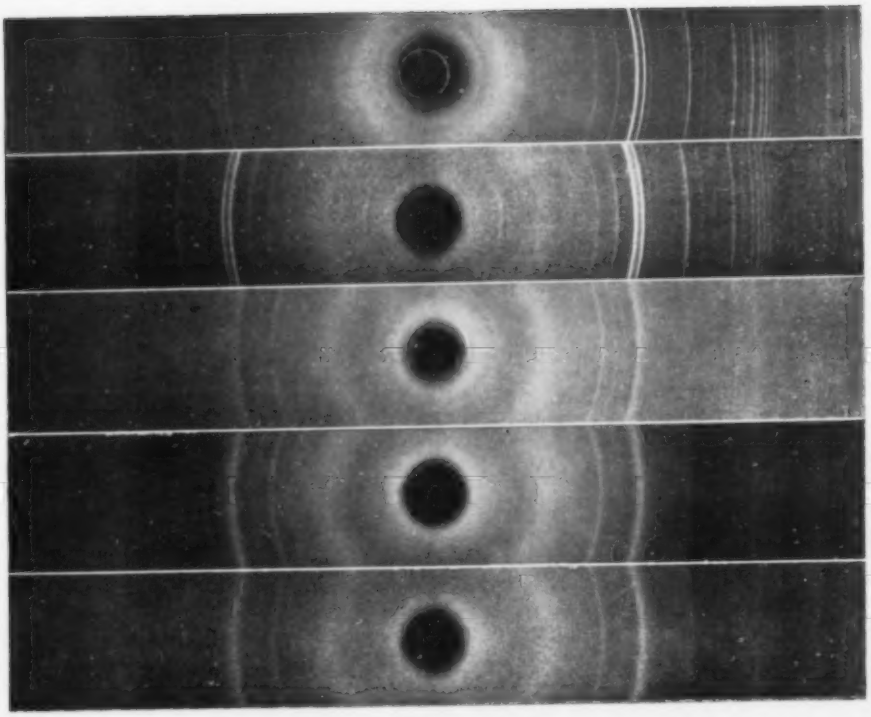

FIG. 1. X-ray diffraction diagrams for several substances. Filtered FeK radiation, $r = 57{\cdot}3$ mm. (a) Francolite. (b) Dental enamel. (c) Oral calculus. (d) *In vitro* calculus. (e) Dentine.

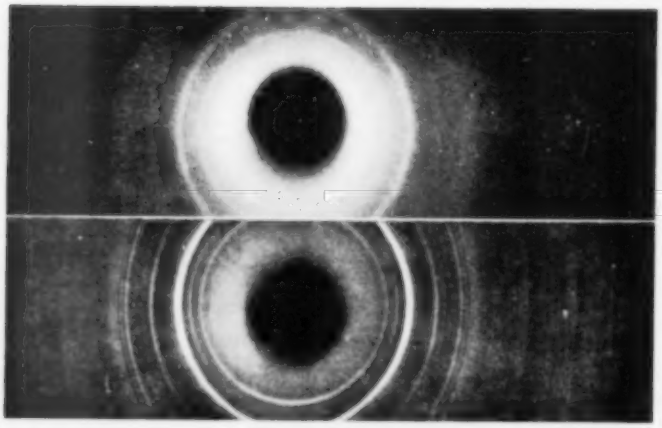

FIG. 2. Comparison of X-ray diffraction diagrams of *in vitro* calculus and fossil dental enamel (dahllite). Filtered Cu*K* radiation, $r = 28{\cdot}7$ mm. (For detailed measurements on spacings and intensities for dahllite see MCCONNELL, 1960a.) (a) *In vitro* calculus. (b) Fossil dental enamel.

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CHARACTERISTICS AND PROPERTIES OF A BOVINE SUBMAXILLARY MUCOID

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Abstract—A purified mucoid has been obtained from the bovine submaxillary glands by chloroform–Naxonate G treatment. The mucoid exhibits electrophoretic homogeneity with a mobility of $-5{\cdot}1 \times 10^{-5}$cm²/V/sec at pH 7·8 and $-7{\cdot}8 \times 10^{-5}$cm²/V/sec at pH 10·3. The compound contains hexosamine, 21·8 per cent (2·84 per cent glucosamine and 18·96 per cent galactosamine), sialic acid, 25·0 per cent, with traces of fucose and uronic acid. The mucoid exhibits a viscosity of 19·9 cP at 0·5 per cent in water and 2·76 cP at 0·5 per cent in 0·2 M phosphate buffer, pH 7·0.

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IN the course of developing a synthetic saliva for studying calculus formation an extract was prepared from bovine submaxillary glands (DRAUS and LEUNG, 1960). We have isolated a mucoid from the extract and have determined its composition.

Isolations of mucoids from salivary glands have been reported by many others (TANABE, 1939; MEYER, 1945; BLIX, SVENNERHOLM and WERNER, 1952; SIMMONS, 1950; PIGMAN and TSUIKI, 1959). In most cases, however, the material obtained was not very soluble in water. Also, since only isolated components were sought, the total composition of the mucoid was usually disregarded.

In this communication we wish to present the method of isolation and the total composition of a soluble mucoid, obtained from the bovine submaxillary gland by a chloroform–Naxonate G treatment, followed by dialysis and lyophilization.

EXPERIMENTAL

Extraction and isolation of gland mucoid

The gland extraction procedure was essentially that reported by DRAUS and LEUNG (1960) except that in the final step the extract solution was not lyophilized, but treated in the manner described below in order to obtain the mucoid.

For the isolation of the mucoid the extract solution from 300 g of submaxillary glands, usually 1 litre, to which had been added 15 g of sodium chloride (HEIMER and MEYER, 1956), was treated with an equal volume of chloroform. The mixture was stirred for 18 hr at 4°C, centrifuged, and the aqueous phase removed. The chloroform layer was washed with a small volume of water, and the washing added to the water removed originally. The stirring with chloroform was repeated three times.

Following the third chloroform treatment 100 g of Naxonate G (a poly-xylene sulphonate, Pedlow–Nease Co., Lock Haven, Pa.; SIMMONS, 1950) was added to the aqueous phase and the solution again stirred for 18 hr at 4°C. The pH of the solution was then adjusted to 4·0 with dilute acetic acid and the precipitate which formed was

removed by centrifugation. The supernatant was readjusted to pH of 7·0 with dilute ammonium hydroxide, dialysed against distilled water for 72 hr, and lyophilized. A yield of between 2 and 3 g was obtained.

Analytical methods and physical measurements

The analytical methods were essentially those used by NISIZAWA and PIGMAN (1959), except in the case of sialic acid. For the sialic acid determination we used the diphenylamine reaction (WERNER and ODIN, 1952). β-Sialic acid was used as the standard. Hexosamine was determined by the ELSON–MORGAN method (1933) with glucosamine as the standard. The method of GARDELL (1953) was used to determine glucosamine and galactosamine. Protein was measured by the Biuret reagent (ROSENTHAL and CUNDIFF, 1956) with serum albumin as the standard. Carbohydrate was determined by the tryptophane–sulphuric acid method (DISCHE, 1949) with glucose as the standard. Fucose was determined by the cysteine–sulphuric acid method (WINZLER, 1955) and uronic acid by the carbazole reagent (DISCHE, 1955) with glucuronic acid as the standard.

The ultraviolet spectrum was determined on a Beckman DK-5 spectrophotometer. The determination was carried out on a 0·04 per cent solution of the mucoid in 0·1M sodium hydroxide using a 0·04 per cent aqueous mucoid solution as a blank.

Optical rotation was determined with a Rudolph model 200 photo-electric polarimeter.

Viscosities were determined with Ostwald pipettes maintained at 37°C in a water bath.

Electrophoretic analysis was done on a Perkin–Elmer model 38, electrophoresis apparatus with Tiselius-type 6 ml cell at pH 7·8 (phosphate–NaCl buffer, ionic strength 0·2) and at pH 10·3 (Na_3PO_4, 0·0069 M; Na_2HPO_4, 0·0051 M; NaCl, 0·0433 M) by Dr. D. PLATT, University of Pittsburgh School of Medicine.

Spectral analyses for elemental composition were carried out by the Analytical Laboratories, Colgate–Palmolive Co., Jersey City, N. J.

Amino acid determinations were by paper chromatography. Ten milligram samples were hydrolysed with 1 ml of 6 N HCl for 18 hr at 100°C in a sealed tube. The solution was filtered and evaporated *in vacuo* in the presence of soda lime. One millilitre of water was added to the residue and six lambda were spotted on Whatman No. 1 paper. Control solutions of the various amino acids were prepared at a concentration of 1 mg per ml. These were run simultaneously with the hydrolysate. The solvent system employed was butanol:acetic acid:water (4:1:5). For the detection of the amino acids both ninhydrin (TOENNIES and KOLB, 1951) and ninhydrin–copper (MOFFAT and LYTLE, 1959) were used.

RESULTS

The product obtained from the lyophilization was a fluffy white powder which was readily soluble in water to 1 per cent. The results for the total composition are presented in Table 1. From the data we can see that the principal components are protein, sialic acid and hexosamine, with indications of a small amount of uronic acid and deoxyhexose.

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TABLE 1. COMPOSITION OF MUCOID

Constituent	% Dry wt.
Ash	2·75
Nitrogen	8·97
Protein (as serum albumin)	38·20
Hexosamine (as glucosamine)	21·80
glucosamine	2·84
galactosamine	18·96
Sialic acid (as β-sialic acid)	25·00
Carbohydrate (as glucose)	6·46
Deoxyhexose (as fucose)	0·65
Uronic acid (as glucuronic)	0·53

Elemental analysis

A spectral analysis on the mucoid indicates the presence of Ca (3·3%); Na (1·1%); P (0·3%); with traces of Cu, Mg and Fe, in addition to C (42·44%); H (6·35%); S (2·44%) and N (8·97%).

Amino acid composition

The amino acid composition of the protein fraction is given in Table 2. The analysis indicates the presence of at least sixteen amino acids. The ninhydrin–copper

TABLE 2. PAPER CHROMATOGRAPHY OF MUCOID HYDROLYSATE (BUTANOL–ACETIC ACID–WATER; 5:1:4)

Amino acid	R_f	
	Known	Hydrolysate
Cystine	0·03	0·03
Histidine	0·07	0·06
Arginine	0·08	0·08
Asparigine	0·10	0·10
Serine	0·12	0·12
Glycine	0·15	0·14
Aspartic acid	0.18	0·17
Threonine	0·21	0·20
Glutamic acid	0·24	0·23
Alanine	0·27	0·27
Proline	0·30	0·30
Tyrosine	0·37	0·36
Valine	0·45	0·46
Phenylalanine	0·54	0·56
Leucine	0·60	0·60
Lysine*	0·08	0·08

* Lysine was shown to be present by a two dimensional chromatogram using butanol–acetic acid–water and 2, 4, lutidine–collodine–water (1 : 1 : 2).

indicator of Moffat and Lytle (1959) made it possible to identify the amino acids by the different colours which they produced. Confirmation was provided by comparison with chromatograms of the known amino acids.

Viscosities

Viscosities of the crude gland extract and the mucoid were determined in both water and phosphate buffer of pH 7·0. The variations in flow times among the different Ostwald viscosimeters appeared to have little effect on the measured relative viscosities. Viscosities were determined in quadruplicate, using different Ostwald viscosimeters, and the readings for each sample averaged. These results are presented in Table 3.

Table 3 Viscosity measurements Ostwald pipettes—37°C.

System	Centipoises	
	Crude	Purified
0·5% in water	2·60	19·90
0·5% in 0·2 M phosphate buffer, pH 7·0	1·67	2·76

U. V. spectrum and optical rotation

The U.V. spectrum gave peak absorption maxima at 217 and 268 mμ. The peak at 268 mμ corresponds to those reported by other authors, which are said to fall between 265 and 280 mμ.

The specific rotation was $[\alpha]\ D^{25} = +\ 5{\cdot}2°$.

Electrophoresis

Fig. 1 illustrates the electrophoresis pattern of the mucoid at pH 7·8 and 10·3. Mobilities based on the ascending boundary were calculated to be $-5{\cdot}1 \times 10^{-5}$ cm²/V/sec at pH 7·8 and $-7{\cdot}8 \times 10^{-5}$ cm²/V/sec at pH 10·3.

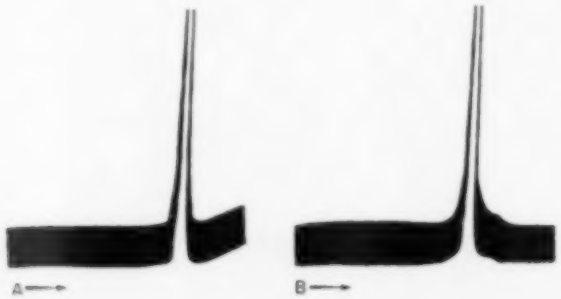

Fig. 1. Electrophoresis pattern; (a) pH 7·8, ionic strength, 0·2, phosphate–NaCl buffer, time 265 min, mobility $-5{\cdot}1 \times 10^{-5}$ cm²/V/sec. (b) pH 10·3, mobility $-7{\cdot}8 \times 10^{-5}$ cm²/V/sec.

DISCUSSION

The submaxillary mucin reported in this work has a sialic acid–galactosamine ratio of 1 if one considers the fact that the galactosamine exists in the acetylated form. This agrees with that reported by NISIZAWA and PIGMAN (1959), HEIMER and MEYER (1956) and ODIN (1958). The hexosamine has both glucosamine and galactosamine in a ratio of approximately 1:6, both in the acetylated form.

The total nitrogen and hexosamine values correspond well to those indicated by MEYER (1945) for a mucoid obtained from bovine submaxillary gland by precipitation, namely, nitrogen 8–9 per cent and hexosamine 18–22 per cent.

In the spectral analysis the traces of Cu, Mg and Fe are probably impurities and not an integral part of the molecule. It is possible that the Na, Ca and P are part of the overall structure.

In the viscosity determination, the low viscosity of the crude extract as compared to the purified material may be explained by the fact that the crude material contains less mucoid. Also, as can be seen in Table 3 the presence of salts drastically affects the viscosity of the purified material. The presence of salt in the undialysed crude material may account, in part, for its low viscosity.

An interesting observation on the amino acid analysis is the fact that most investigators have indicated the absence of sulphur-containing and aromatic amino acids in mucoids (PIGMAN and TSUIKI, 1959). We have been able to demonstrate the presence of both. At present we are in the process of determining quantitatively the amino acid composition. These results will be reported at a future time.

The specific rotation of the compound has caused us some concern. The small positive rotation leads us to suspect mutarotation. This possibility was investigated by allowing the compound to stand in the polarimeter for a period of 4 hr and noting any change in rotation. No change was observed over this period of time.

The electrophoretic pattern indicates homogeneity at pH 7·8 and 10·3 since only one component is visible after a period of 265 min. NISIZAWA and PIGMAN (1959) reported a mobility of -5×10^{-5} cm^2/V/sec for the major component (at pH 7·8, phosphate =NaCl buffer, ionic strength 0·1) of mucin C obtained from the bovine submaxillary gland (two other components were also reported). It is possible that our mucoid, with a mobility of $-5{\cdot}1\times10^{-5}$ cm^2/V/sec is similar to this component.

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STUDIES ON AGGLUTINATION AND HAEMOLYSIS OF SHEEP ERYTHROCYTES BY LACTOBACILLUS ANTISERUM

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Abstract—Sheep erythrocytes, modified by exposure to soluble antigens extracted from a strain of *Lactobacillus acidophilus*, agglutinate in the presence of antiserum prepared by injection of the untreated bacterial cells. The red blood cells haemolyse when complement is added to this system. The soluble antigen was extracted by use of N/20 HCl on whole bacterial cells. Erythrocytes from seven kinds of animals were tested to determine that sheep red blood cells were the most suitable. The haemolytic test was more sensitive than either bacterial agglutination or sheep erythrocyte agglutination. Bacterial agglutination was more sensitive than sheep haemagglutination.

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INTRODUCTION

A NUMBER of workers since HIRST (1941) have reported that erythrocytes modified by various bacterial and viral antigens agglutinate in the presence of antibodies prepared against the cells or viruses. Such haemagglutination, called indirect microbial or passive microbial haemagglutination, is apparently more sensitive than the conventional bacterial or viral agglutination. GAINES and LANDY (1955) detected antibodies against *Pseudomonas aeruginosa* in normal human sera and NETER *et al.* (1955) discovered antibodies against enteropathogenic *Escherichia coli* in the sera of patients with epidemic diarrhoea by using haemagglutination techniques. The bacterial agglutination tests were negative in both instances. NETER (1952) conducted a number of experiments comparing haemagglutination, haemolysis and bacterial agglutination in *E. coli* serogroup OIII and found that the haemolytic test on sheep erythrocytes modified by exposure to OIII antigen showed the highest serum titre. These studies also indicated that the species of the red blood cells used in the tests were responsible for the variations in the results.

The purpose of this paper is to present the results of our studies on extraction of antigens from a strain of *Lactobacillus acidophilus*. The serologic reactions of these antigens in haemagglutination and haemolytic tests of modified sheep erythrocytes were compared with homologous bacterial cell agglutinations.

MATERIALS AND METHODS

Bacterial antigens

Lactobacillus acidophilus ATCC No. 11506 was grown and maintained in SL medium of ROGOSA, MITCHELL and WISEMAN (1951) at a pH of 5·4 in the absence of agar.

Bacterial suspensions were prepared after washing 48 hr cultures three times in buffered saline, pH 7·0 made up as follows: Na_2HPO_4, 5·7 g; KH_2PO_4, 3·63 g; NaCl, 4·53 g; water to 1000 ml (RANTZ, RANDALL and ZUCKERMAN, 1956). One loopful of the packed cells (approximately 2 mg) was suspended in 2 ml of buffered saline, then 0·5 ml of this suspension was added to each tube of diluted antiserum.

Antigen A was obtained after the application of a modification of the Lancefield extraction procedure (LANCEFIELD, 1933; SHARPE, 1955) to about 1·75 g (wet wt.) of washed cells. The cells were suspended in 5 ml physiologic saline containing HCl to a final concentration of N/20. The suspension was placed in boiling water for extraction and then centrifuged. The supernatant was neutralized with N NaOH and the precipitate removed by centrifugation; the supernatant was called antigen A and was stored at −20°C until used. Aliquots of this extract were exposed to trypsin, final concentration 0·2 mg/ml, for 1 hr at pH 7·0 at 37°C. The enzyme was inactivated by heating at pH 3·0 for 3 min at 60°C.

Antigen B was obtained by suspending about 1·75 g of washed, packed cells in 15 ml of buffered saline pH 7·0 and exposing to sonic vibration at 9 kc/s for 2 hr in a Raytheon sonic oscillator at a plate voltage of 110. Centrifugation was conducted at 12,000 rev/min for 15 min; the supernatant was recentrifuged and labelled antigen B.

Antigen C was a culture filtrate suggested by the work of RANTZ, ZUCKERMAN and RANDALL (1952). The supernatant, after removal of cells from the SL medium, was adjusted to pH 7·0 and dialysed overnight at 4°C against isotonic saline. The dialysate was sterilized by Seitz filtration and then stored at −20°C.

Immunization

Blood was obtained by heart puncture from four male rabbits weighing about 2·5 kg, the serum was separated from the clotted blood, tested for agglutinins with the whole cell antigen, then pooled.

The injection schedule for preparation of antiserum was similar to that used by WILLIAMS and FRANCK (1957). The rabbits received 0·2 ml of concentrated vaccine daily for four consecutive days the first week, 0·5 ml for the same number of days the second week and 1 ml for four consecutive days in the third week. The animals were trial bled 7 days after the last injection; if the titre was unsatisfactory the schedule used in the third week was repeated. All antisera were pooled and stored after heating to 56°C for 30 min, no preservative was added.

Absorption of antisera for serologic tests

All normal sera and antisera were absorbed with sheep erythrocytes in order to remove heterologous antibodies (NETER, 1956). Rabbit serum was diluted 1:10; to 20 vol of the diluted serum 1 vol of packed, washed, sheep erythrocytes was added and suspended. This was centrifuged at 3000 rev/min for 10 min after standing 10 min. The procedure was immediately repeated without discarding the sediment. The supernatant was used as absorbed rabbit serum. This procedure was repeated using six other species of erythrocytes.

Erythrocytes were obtained from seven animal species and preserved in Alsever's solution (MIDDLEBROOK, 1950) for not longer than 2 weeks. The cells were washed

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three times in buffered saline and then suspended in enough saline to make the turbidity equal to an optical density at 550 mμ; this was equal to 1·5 on a Hidachi EPO type-B spectrophotometer. The concentration adopted is equal to 0·5% by volume and contains about 8×10^4 cells/ml.

Complement

Blood was obtained by heart puncture from several healthy guinea pigs and the separated serum was pooled. Antibody for erythrocytes was removed by absorption of 15 vol of complement, diluted 1:3, with 1 vol of packed cells for 10 min. The cells were removed by centrifugation at 5000 rev/min for 5 min, then the absorption was repeated two more times. The above procedures were performed at 3°C.

Titration followed a modified Kolmer method (GRADWOHL, 1956). A 2% suspension of sheep erythrocytes sensitized with 5 units/0·25 ml of haemolysin was prepared. Diluted complement was added to determine the minimum dose showing complete haemolysis (minimum haemolytic dose = 1 unit).

TABLE 1.

	Test tube				
	1	2	3	4	5
Complement (1:100) (ml)	0·45	0·40	0·35	0·30	0·25
Saline (ml)	0·30	0·35	0·40	0·45	0·50
Modified red cells 2% (ml)	0·25	0·25	0·25	0·25	0·25
Incubate 30 min at 37°C					
Haemolysis	+++	++	−	−	−

− = no haemolysis. +++ = complete haemolysis.

The results given in Table 1 indicate that 0·45 ml of 1:100 complement is the titre.

Serologic tests

Bacterial agglutination. The tests were carried out using 0·5 ml of serially diluted sera and 0·5 ml of the bacterial suspension described above. The mixtures were held in a water bath at 37°C for 2 hr, refrigerated overnight and read by means of an agglutinoscope. The titre, the highest dilution showing partial agglutination, was 1:3200.

Haemagglutination. Erythrocytes were modified by the methods of NETER (1952) and RANTZ *et al.* (1956). An equal volume of 0·5% by volume of blood cells was added to each dilution of soluble antigen and incubated for 1 hr at 37°C. The cells were washed three times with buffered saline and then suspended in saline to a concentration of 0·5%. The modified cells were added in 0·5 ml amounts to equal volumes of the serially diluted sera in 10×100 mm test tubes and incubated in a water bath at 37°C for 1 hr, with occasional shaking. The tubes were refrigerated overnight and read by the pattern technique of DENNY (1953).

Haemolysis. This was demonstrated by adding 0·05 ml of 1 : 10 dilution of complement to each tube prepared as just described, but not incubated. These tubes were

then incubated 1 hr at 37°C, centrifuged and read for haemolysis. The titre was read as the reciprocal of the initial serum dilution. The dose of complement was twice the minimum haemolytic dose (i.e. diluted half as much).

RESULTS

The agglutination of sheep erythrocytes, modified by the several soluble lactobacillus antigens, in rabbit antisera indicated that antigen A was more closely related to antigens in the bacterial cells than either antigens B or C. The results are presented in Table 2. Even though the preparation of these antigens does not allow quantitative comparisons there appears to be a sharp difference in the effects on sheep erythrocytes. The highest titre observed in diluted antiserum was 1 : 320. However, erythrocytes exposed to antigen A diluted 1:8 were still clearly agglutinable. Thus all further work was centred on antigen A.

TABLE 2. COMPARISON OF BACTERIAL HAEMAGGLUTINATION BY THREE ANTIGENS ACTING ON SHEEP RED BLOOD CELLS FOR 60 min

Anti-bacterial rabbit serum	Antigen A			Antigen B			Antigen C		
	1:1	1:2	1:4	1:1	1:2	1:4	1:1	1:2	1:4
1:40	+++	+++	+++	+++	++	+	±	−	−
1:80	+++	+++	+++	++	+	−	−	−	−
1:160	+++	+++	+++	−	−	−	−	−	−
1:320	+++	+++	+++	−	−	−	−	−	−
Normal rabbit serum 1:40	−	−	−	−	−	−	−	−	−

− = no haemagglutination. + ~ +++ = various grades of haemagglutination.

Our next concern was to determine whether the kind of erythrocytes employed in the tests could alter the results. Table 3 shows the results using antigen A diluted 1:4 and erythrocytes from seven different animals. It is apparent that sheep erythrocytes are the most suitable for the adsorption of the antigen.

TABLE 3. HAEMAGGLUTINATION OF RED BLOOD CELLS OF VARIOUS SPECIES TREATED WITH ANTIGEN A

Anti-bacterial rabbit serum	Sheep	Rat	Dog	Human	Guinea pig	Rabbit	Ox
1:40	+++	+++	+++	+++	+++	++	−
1:80	+++	+++	++	++	++	++	−
1:160	+++	+	+	+	++	±	−
1:320	+++	−	−	−	−	−	−
Normal rabbit serum 1:40	−	−	−	−	−	−	−

Note: In modification of all species of blood cells, 1:4 diluted antigen A was used.
− = no haemagglutination. + ~ +++ = various grades of haemagglutination.

Table 4 presents the results of the effects of time on antigen adsorption when the antigen was diluted. It is obvious that extension of time allowed greater adsorption of antigen, but the greatest effect was observed when the concentration was highest. Antigen diluted 1:6 and exposed for 45 min allowed agglutination with antiserum diluted 1:320. Greater dilutions were less effective.

TABLE 4. TITRATION OF ANTIGEN A AND THE EFFECTS OF TIME ON THE ABSORPTION OF THIS ANTIGEN ON SHEEP RED BLOOD CELLS

Anti-bacterial rabbit serum	Antigen dilution								
	1:6			1:30			1:60		
	Modification time on red blood cells (min)								
	5	15	45	5	15	45	5	15	45
1:10	+++	+++	+++	+	++	++	−	+	+
1:20	+++	+++	+++	+	++	++	−	−	+
1:40	+++	+++	+++	−	+	++	−	−	+
1:80	+	++	++	−	+	+	−	−	−
1:160	+	+	++	−	−	+	−	−	−
1:320	−	−	+	−	−	−	−	−	−
Normal rabbit serum 1:10	−	−	−	−	−	−	−	−	−

Haemolysis of erythrocytes modified by exposure to antigen A, then exposed to rabbit antiserum and complement is shown in Table 5. The activity of absorbed and unabsorbed complement is shown to demonstrate the importance of pre-absorption with erythrocytes. The highest dilution of serum in which haemolysis took place was 1:100,000. This was about 300 times the sensitivity of haemagglutination and about thirty times the titre in bacterial agglutination as is seen in Table 6.

TABLE 5. TITRATION OF HAEMOLYSIS AND EFFECT OF NORMAL ANTIBODY OF COMPLEMENT

Anti-bacterial rabbit serum	Absorbed complement (1:10)		Untreated complement (1:10)	
	Modified red cells	Untreated red cells	Modified red cells	Untreated red cells
10^{-2}	+++	−	+++	+++
10^{-3}	+++	−	+++	+++
10^{-4}	+++	−	+++	+++
10^{-5}	+	−	+++	+++
5×10^{-5}	−	−	+++	+++
Normal rabbit serum 10^{-2}	−	−	+++	+++

− = no haemolysis. +++ = complete haemolysis.

Table 6. Comparison of titres of the immune reactions

Reactions	Maximum dilution of antiserum
Conventional bacterial agglutination	1:3200 (agglutinative titre)
Bacterial haemagglutination	1:320 (agglutinative titre)
Haemolytic modification	1:100,000 (haemolytic titre)

DISCUSSION

A principal difficulty in studies of agglutination of lactobacilli is the tendency for these bacteria to agglutinate spontaneously. Orland (1950) reported several ways to obviate this interference. However, Aiba (1958) suggested that suitable suspensions could be obtained by controlling the pH during and after washing with physiologic saline. Adjustment to a pH of 7·2 resulted, after standing overnight, in an even suspension. These difficulties stimulated a search for some other method for serologic study of lactobacilli.

The use of the haemagglutination and haemolytic system seemed to hold promise but a soluble antigen was needed. Thus lactobacilli were extracted with HCl and other methods were used. It was apparent from our tests that antigen A was the most useful product. It is an extremely crude product and we only have indirect evidence suggesting that it is a polysaccharide. It is heat-stable in acid (pH 3·0) solution and resists tryptic digestion. Most antigens useful in haemagglutination and haemolysis are reported to be polysaccharides (Amies, 1951; Chen, 1952; Silverman, 1954). It is interesting that if HCl is used in a concentration greater than N/20 the bacterial cells became an amorphous mass and the titre of antigen decreased. The low concentration did not lead to destruction of the bacterial cells.

Antigens A, B and C were not prepared from comparable quantities of material, thus there is a possibility that the amounts of the antigens in the respective preparations differ markedly. However, the results of tests comparing antigen reactivity, indicated that antigen A was highly reactive and thus worthy of further study. The marked adsorption of antigen A by sheep erythrocytes, in contrast to erythrocytes from other animal species, is of particular interest. There was no indication of an explanation for such a great difference.

Our results with haemagglutination indicate it to be a less sensitive method than bacterial agglutination. This is in contrast to results reported by Gaines and Landy (1955) and Neter *et al.* (1955) for *Pseudomonas aeruginosa* and *Escherichia coli* respectively. However, they do agree with the report of Aiba (1958) who extracted an antigen from *Bacillus bifidus* using neutral saline and heat. The data from the haemolysis of modified erythrocytes suggest an extremely sensitive method for studying the antigenic structure of lactobacilli. It is about thirty times more sensitive than bacterial agglutination when measured in terms of serum dilution. Thus it should be most useful in studying major antigens which may not be separable at lower dilutions of antiserum.

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Studies of these techniques are being continued to determine the presence of lactobacillus antibodies in human sera and the nature of the soluble antigen.

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ELECTROPHORESIS OF SYNTHETIC HYDROXYAPATITE

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Abstract—The ζ potential of a synthetic hydroxyapatite is calculated from measurements of the electrophoretic mobility using a "Northrop–Kunitz Cataphoresis Apparatus" under various conditions affecting its solubility at constant pH, 7·40, and ionic strength, 0·165. The ζ potential varies with the solid: solution ratio of the sample, being negative in each instance and tending at higher ratios towards a limiting value of 1·5 mV. Excess calcium and phosphate in the solution produce a positive and negative ζ potential respectively. There is a linear relationship between the ζ potential and the logarithm of the final calcium: phosphate ratio of ions in solution, the zero ζ potential occurring when this ratio is equal to the ratio of these ions in the unit cell of hydroxyapatite. Citrate produces a larger negative ζ potential than an equivalent molar concentration of phosphate.

INTRODUCTION

THIS is a report on the electrokinetic properties of a sample of synthetic hydroxyapatite in relation to factors which affect its solubility and physiochemistry.

Physiochemistry of hydroxyapatite

Many of the chemical and physiological properties of bones and teeth may be explained by the small crystal size and consequent large surface area to mass ratio of the crystals in the mineral phase. As a result of this large ratio the predominant reactions undergone by the crystals are general surface phenomena, i.e. ionic exchange (iso and hetero), adsorption and resorption of ions. As a result of this small crystal size an appreciable fraction of the total crystal mass is composed of surface ions and there is no rigorous stoichiometric balance of ions at the crystal surface such as occurs in the centre of a large crystal. Hence, a large fraction of hydroxyapatite is composed of surface ions resulting in a series of chemical compounds of variable composition and properties, all of which may be classified as hydroxyapatites and all of which have similar X-ray diffraction and electron diffraction patterns. These techniques do not detect the variations that may occur in surface composition. As the activity of the solid is not a constant the mineral does not have a constant solubility in aqueous solution. This variable solubility of hydroxyapatites in bones and teeth (impure hydroxyapatites) has been established in relation to changes in pH, ionic strength, composition of the liquid in which they are bathed and with changes in the solid to solution ratio of the mineral and solution (NEUMAN and NEUMAN, 1958, p. 23; LEACH, 1959).

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Electrokinetics

When a particle is placed in aqueous solution there is a separation of charges, one surface becoming positively charged with respect to the other. The electrical potential difference set up between the particle and the bulk solution will cause the particle to move through the liquid under the influence of an external electrical field. This is one particular manifestation of a more general phenomenon known as "electrokinetics" and is concerned with the motion of two charged surfaces relative to each other. The motion of a particle in solution under an applied electrical field is known as electrophoresis or, as it was once called, cataphoresis (BULL, 1951).

Ionic crystals in aqueous solution show electrokinetic effects which are specifically determined by the structure and chemical composition of the crystal. Certain of these effects arise through preferential adsorption or release at the crystal surfaces of ions compatible with the crystal lattice and these effects are due to the operation of the same forces as are responsible for crystal breakdown and growth. Ions foreign to the lattice may also be preferentially taken up by the crystal surfaces or exist at an increased concentration in the layer of relatively fixed and orientated ions surrounding the crystal. One effect of these ions, which in turn will influence the interaction of the crystals with other ions, can be shown by measuring the electrophoretic mobilities of the crystals under the various conditions mentioned above.

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MATERIALS AND METHODS

Electrophoresis

The electrophoretic mobility was found by measuring the velocity of the individual particles suspended in aqueous solution when enclosed in a small flat hollow cell across which an electrical potential difference was applied. The movement of the particles was observed directly through a microscope containing a micrometer eyepiece.

It is not intended to go into details of the general experimental technique and theory of electrophoresis as these are presented at length elsewhere (MACINNES, 1939; GORTNER and GORTNER, 1949; BULL, 1951; STERN, 1956). The determinations were carried out using a "Northrop–Kunitz Cataphoresis Apparatus" as suggested by MUDD and MUDD (1929) and modified by the use of copper–copper sulphate electrodes to prevent the evolution of gas at the electrodes. The potential gradient in the cell was obtained from determinations of the cell dimensions, the current flowing in the circuit and the conductivity of the solution. Measurements of particle velocity were carried out at 25°C using a potential gradient in the cell as near to 2 V/cm as possible. The value of the mobility at 21 per cent from the bottom of the cell was taken as the electrophoretic mobility (ABRAMSON, 1931). At least twenty determinations were made on each solution with the current flowing alternately in each direction and the mean of these taken as the mobility. The observed mobility was independent of the size and shape of the particles and mean determinations from different aliquots of the same flask and from duplicate flasks agreed to within 5 per cent.

To facilitate comparison with other work the electrophoretic findings are reported in terms of the ζ potential and are calculated from the Henry–Smoluchowski equation in the form

$$\zeta = \frac{4\pi\eta u}{ED} f(b)$$

where η is the viscosity and D the dielectric constant of the solvent (taken in this case as the bulk values for water at 25°C), E the applied potential field in volts per centimetre, U the electrophoretic mobility and b a parameter representing the ratio of the linear dimensions of the particle to the thickness of the electrical double layer surrounding the particle and which under the present circumstances may be taken as unity (HENRY, 1931). At a potential gradient of 1 V/cm the above equation resolves to $\zeta = 12{\cdot}85U$ where ζ is expressed in millivolts and U in microns per second. A positive ζ potential indicates that the particle migrates towards the cathode and a negative ζ potential towards the anode. The following results are presented in terms of ζ expressed in millivolts.

Procedure

Except for the hydroxyapatite, which was part of an otherwise well characterized sample (L apatite—WEIKEL, NEUMAN and FELDMAN, 1954; NEUMAN and NEUMAN, 1958), chemicals of analytical reagent grade were used. All solutions were buffered with 0·02 M diethylbarbituric acid, adjusted to pH 7·40 with KOH and to a final ionic strength of 0·165 with KCl. Each suspension of hydroxyapatite was shaken for 10 days or more at 25 $\pm$ 2°C in the presence of $CHCl_3$ (5 ml/l) to minimize bacterial growth. This time period had previously been found to give a reproducible end point, within experimental error, in solubility studies of hydroxyapatite (LEVINSKAS and NEUMAN, 1955). An aliquot of the suspension was then centrifuged, (r.c.f. 1200 *g*), for an empirical length of time varying from 45 sec to 5 min until only the smaller aggregates of crystals were left in suspension, and the supernatant solution was carefully poured off. This solution was used for determining the electrophoretic mobility and contained less than 0·1 per cent by weight of the hydroxyapatite suspension. An empirical centrifugation time was used for each solution as the rate of sedimentation varied considerably between one suspension and another because of the different electrical states of the particles.

Analytical methods

All solutions to be analysed were first filtered through molecular filter membranes (Millipore Filter Corporation, 470 mμ size). Calcium was estimated either by flame photometry (TORIBARA, DEWEY and WARNER, 1957) or by a titration method with ethylenediaminetetracetic acid, using Eriochrome black T as indicator (SOBEL and HANOK, 1951). Phosphorus was estimated by Chen's modification of the method of Ammon and Hinsberg (CHEN, TORIBARA and WARNER, 1956) and citrate by using ^{14}C labelled citrate, the decay being measured by a scintillation technique. All analytical procedures were carried out in duplicate or triplicate and agreed to within 5 per cent in all cases. The mean results are presented in the following Figures and Tables.

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TABLE 1. SOLUBILITY AND ζ POTENTIAL OF HYDROXYAPATITE SHAKEN AT DIFFERENT SOLID:SOLUTION RATIOS FOR 14 DAYS AT 25°C IN BUFFER SOLUTION pH 7·40 AND IONIC STRENGTH 0·165

Solid:solution (g/l)	Solubility (M × 10^4)		ζ potential (negative)
	Calcium	Phosphate	
0·05	0·92	0·53	16·6
0·1	1·49	0·90	10·9
0·5	1·68	1·13	4·6
1·0	1·70	1·17	3·8
5·0	1·90	1·44	2·1
10·0	2·03	1·60	1·8
25·0	2·30	1·75	1·6
30·0	2·54	1·98	1·5
40·0	2·60	2·10	1·4

RESULTS AND DISCUSSION

Solid : solution ratio

The ζ potential at solid: solution ratios from 0·05–40 g/l are shown in Fig. 1, and Table 1 shows the solubility of the sample under these conditions. The ζ potential and the solubility varied, the former being negative in each case, and tending at the higher solid : solution ratios towards a limiting value of the order of 1·5 mV.

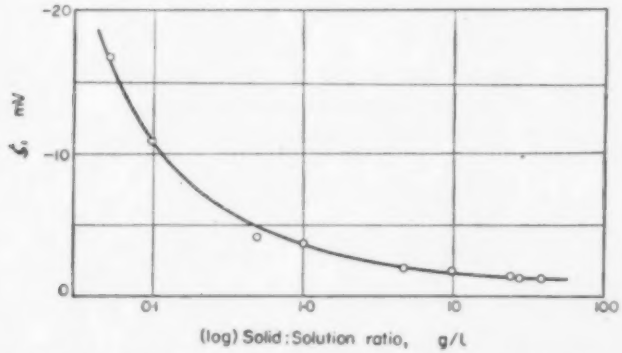

FIG. 1. ζ potential of hydroxyapatite shaken at different solid : solution ratios for 14 days at 25°C in buffer solution, pH 7·40 and ionic strength 0·165.

The variation in ζ potential with changes in the solid : solution ratio was to be expected since the solubility is a function of this ratio and the ζ potential is in turn dependent on the release or adsorption of ions at the crystal surfaces. As a result, further work with the mineral matter of bones and teeth will apparently necessitate strict control of the solid : solution ratio, particularly so as the rate of change of ζ potential is greatest at the smaller ratios, and which, for work of the greatest accuracy, precludes the use of small amounts of mineral because of the relatively large volume

of liquid required for the electrophoresis cell to operate effectively ($\geq$25 ml). However, one possible use of this technique may be as a sensitive method for detecting small differences between one hydroxyapatite sample and another, for although in the present instance a 400-fold change in solid : solution ratio produced only a three- to four-fold change in solubility, there was over a ten-fold change in the ζ potential.

The relationship between the changes in ζ potential and the changes in solubility at different solid : solution ratios is a function of the relative amounts of mineral dissolved per unit weight of material and the relative amounts of each particular ion released at each solid : solution ratio. As the samples were in an environment of constant pH, and hence constant hydroxyl ion concentration, the effect of the hydroxyl ions on the ζ potential was the same in each case and was superimposed on the effects produced by the different solubilities of the calcium and phosphate ions at each solid : solution ratio. Although the solubility of the samples increased with respect to both calcium and phosphate with increase in solid : solution ratio (Table 1), the amount of mineral dissolved per gram of sample decreased, and hence the preferential release of either ion had its greatest effect at the lower solid : solution ratios. The calcium : phosphate ratio of the ions released at the smaller solid : solution ratios (1·24 at 40 g/l), together with the relative amounts of material dissolved per unit weight of sample, explains the shape of the ζ potential–solid : solution ratio curve in Fig. 1. Although at the higher ratios phosphate was apparently preferentially released from the sample, the presence of a constant excess of hydroxyl ions and the relatively small amount of mineral dissolved per unit weight of sample could account for the continued negative ζ potential even though a preferential release of excess phosphate ions might tend to suggest a positive potential.

Common ion effects

Since crystals may acquire a ζ potential by adsorption of ions at their surfaces and hydroxyapatite is known to take up calcium and phosphate from solutions containing excess of these ions, it is to be expected that the presence of excess calcium or

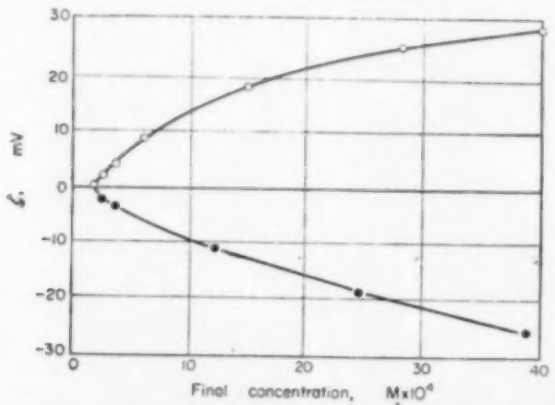

FIG. 2. ζ potential of 25 g/l amounts of hydroxyapatite shaken for 14 days at 25°C in buffer solution, pH 7·40, ionic strength 0·165, in the presence of excess calcium (○) or phosphate (●).

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phosphate ions in the solution would result in a more positive or negative ζ potential respectively. The effect on the ζ potential of different amounts of excess calcium ($CaCl_2$), and phosphate (KH_2PO_4 and K_2HPO_4) in solution when shaken with 25 g/l amounts of hydroxyapatite is shown in Fig. 2. As could be predicted, excess calcium and phosphate produced positive and negative ζ potentials respectively. A linear relationship was found to exist between the ζ potential and the logarithm of the final concentration of either ion in solution, thus

$$\Delta\zeta = K\Delta\log C$$

where K is a constant and C is the final concentration of ions in solution. A similar relationship has been found to hold for barium sulphate (RUYSSEN, 1940; BUCHANAN and HEYMANN, 1949), with the crystal ions as adsorbable electrolyte. A linear relationship also existed between the ζ potential and the logarithm of the final molar calcium: phosphate ratio of the ions in solution (Fig. 3). It is interesting to note that the position of zero ζ potential occurred at a final calcium : phosphate ratio of ions in solution similar to that of the calcium : phosphate ratio of ions in the unit cell of hydroxyapatite (1·67).

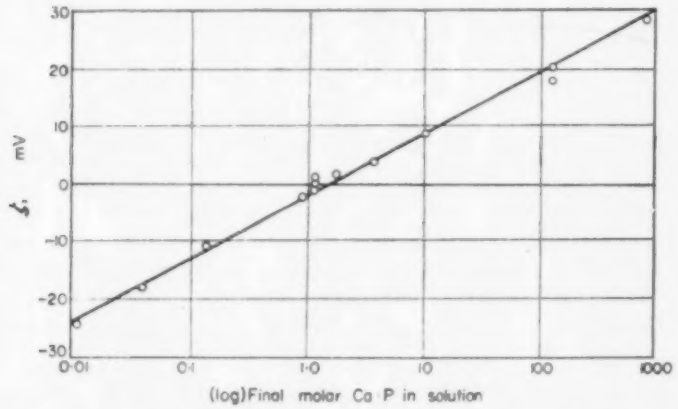

FIG. 3. ζ potential at different final molar calcium : phosphate ratios of ions in solution on shaking 25 g/l amounts of hydroxyapatite for 14 days at 25°C in buffer solution, pH 7·40, ionic strength 0·165, in the presence of excess calcium or phosphate.

An initial excess of ions gave values of the ζ potential which were almost independent of the solid : solution ratio of the samples; an initial excessof calcium (25×10^{-4}M) at ratios of 0·1 and 25 g/l gave positive ζ potentials of 19·1 and 17·8 mV respectively.

Foreign effects—citrate

Since DICKENS (1941) first demonstrated the presence of citrate in bone, there has been a continuing interest in the possibility that it is one of the agents in the homoeostatic mechanisms between bone and serum calcium and phosphate. It has recently been shown that although the parathyroid influences the local production of citric acid in bone in a manner that is not yet clear, it is clear that variation in citric acid

production regulates the local solubility of the bone mineral in the extracellular fluids (MARTIN *et al.*, 1958). Also it has been shown more recently (HARTLES and LEAVER, 1960) that a citric acid–peptide complex occurs in dentine. It may be that the organic citrate ion, which apparently has an affinity for both the organic and inorganic fractions of calcified tissues, has also an important role in the initial deposition of mineral salts at specified sites along an organic fibre of collagen.

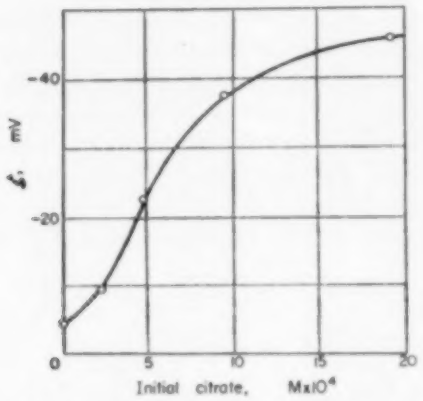

FIG. 4. ζ potential of 5 g/l amounts of hydroxyapatite shaken for 14 days in buffer solution, pH 7·40, ionic strength 0·165, in the presence of different amounts of citrate.

The ζ potential of 5 g/l amounts of hydroxyapatite in the presence of different amounts of citrate is shown in Fig. 4, and Table 2 shows the citrate taken up and the solubilities in each case. The ζ potential became more negative in the presence of citrate and the solubility of the samples increased much more with respect to phosphate than to calcium. Citrate, because of its large size and spatial distribution of atoms will almost certainly have to reside on the surface of the hydroxyapatite crystals and its presence there will account for the negative ζ potential. Larger ions are, in general, less hydrated than smaller ions of similar charge (polarizability) and less work is

TABLE 2. CITRATE TAKEN UP, CALCIUM AND PHOSPHATE RELEASED AND ζ POTENTIAL OF 5 g/l AMOUNTS OF HYDROXYAPATITE SHAKEN FOR 14 DAYS AT 25°C IN BUFFER SOLUTION, pH 7·40, IONIC STRENGTH 0·165 IN THE PRESENCE OF DIFFERENT AMOUNTS OF CITRATE

Citrate (M × 10⁴)			Solubility (M × 10⁴)		ζ potential (negative)
Initial	Final	Taken up	Calcium	Phosphate	
0	0	0	1·96	1·43	4·0
2·37	0·88	1·49	1·89	1·92	9·6
4·75	1·88	2·87	1·87	2·55	22·5
9·48	5·42	4·06	1·91	3·90	37·6
19·00	14·47	4·53	2·16	4·75	45·8

required for them to move into the region of the crystal interface and hence they have a tendency to reach an average position closer to the crystal than do smaller ions. This may account for the fact that a final concentration of citrate in solution produced a larger negative ζ potential than an equivalent final phosphate concentration (c.f. Fig. 2 and Table 2) as citrate, being the larger ion, is able to move more effectively into the electrical double layer surrounding the crystals and this layer is responsible for the observed electrokinetic effects.

General inferences

Although a great deal of experimental work has been carried out, the precise nature and location of the balancing charge and electrokinetic potential (ζ potential) is not known for the system ionic crystal–electrolyte solution in general. However, a theoretical treatment of a simple binary salt (AgBr) that, unlike hydroxyapatite, has a constant solubility product, does predict with some difficulty that the electrical state of the crystals influences the dynamics of the exchange processes occurring at the crystal surfaces (GRIMLEY, 1950). It is not possible to use these methods for a relatively complicated substance such as hydroxyapatite; the results are interesting, however, as they show that the dynamics of the crystal surfaces are influenced by the electrical state of the crystals. Working with hydroxyapatite from a completely different approach, using radioisotope techniques to measure the exchangeable calcium and phosphate, NEUMAN and NEUMAN (1958, p. 75) concluded that "Apparently, the electric potential barrier is the most important variable affecting the rate of exchange." The chemistry of hydroxyapatite is essentially a problem of surface chemistry, and as such the electrical states of the particles must be of prime importance. Although very little can be explained at present, these investigations show that the electrical state of the crystals could be varied in both sign and size under a variety of conditions.

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GINGIVAL HEALTH AND DENTAL CLEANLINESS IN ENGLISH SCHOOLCHILDREN

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University of Leeds Dental School and
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Abstract—3094 children from Leeds, Liverpool and East Anglia were examined and classified according to standards of gingivitis and dental cleanliness. A preliminary test in which the four examiners classified the same 100 children showed that differences between examiners would invalidate comparisons between the three areas. Internal comparisons were made showing that there was a significant association between gingivitis and dental cleanliness in children of all areas; that in 12 year old children the incidence of good gingival conditions and good dental cleanliness was significantly higher in girls than in boys; and that there was a tendency for boys to deteriorate in gingival health between 11 and 12 years, while girls tended to improve between these ages.

One examiner classified standards of gingivitis in 296 children aged 11 and 12 residing in a high fluoride area of East Anglia and 396 similar children in a low fluoride area. No general difference was found in the incidence and severity of gingival conditions between the areas. Where significant differences occurred the children living in the high fluoride area showed better gingival health.

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INTRODUCTION

THE incidence of gingivitis in children has been studied by a number of workers using varied techniques of obtaining data, and many different standards of diagnosis and classification.

In the U.S.A. reports include those of MESSNER *et al.* (1936), BRUCKNER (1943), MASSLER, SCHOUR and CHOPRA (1950), MASSLER, COHEN and SCHOUR (1951), STAHL and GOLDMAN (1953), SAVARA (1955) and RUSSELL (1957a).

In Britain only a few investigations have been done in this field. The largest randomly-selected group was that examined by AINSWORTH and YOUNG (1925) which consisted of 4063 schoolchildren aged from 2 to 14 years. Other reports include those of KING (1945), RUBRA (1948), GOOSE and POTTER (1956), PARFITT (1957) and PARFITT, JAMES and DAVIS (1958).

In the first part of the present investigation children aged 11 and 12 years living in three different areas of England were examined in respect of their gingival state and their standard of dental cleanliness. The objects of the investigation were:

1. To ascertain whether, using simple standards of diagnosis, the four different examiners could arrive at comparable findings.

2. If so, to compare the incidence of gingivitis and dental cleanliness standards in Leeds, Liverpool and East Anglia.

3. To determine the difference, if any, between boys and girls in standards of gingivitis and dental cleanliness.

4. To determine the difference, if any, between 11 and 12 year old children.

5. To ascertain whether a relationship exists between gingivitis and dental cleanliness.

In addition to the general investigation on gingivitis in children, the relationship between gingivitis and fluoride intake has been studied.

MASSLER and SCHOUR (1951) found a slight association between endemic fluorosis and the incidence of gingivitis in Italian children and adults living in high fluoride areas in Italy. BROWN *et al.* (1954) and RUSSELL (1957b) found no relationship between the incidence and severity of gingivitis and the presence of fluorides in the water.

The purpose of the second part of the present investigation was to determine whether children living in a high fluoride area have different rates of gingivitis from those living in areas with a low level of fluoride in the water.

MATERIAL AND METHOD

For the general investigation 3094 secondary modern schoolchildren aged 11 and 12 years were examined, 1750 girls and 1344 boys. The composition of the sample is set out in Table 1.

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TABLE 1. COMPOSITION OF MAIN SAMPLE

Area	Age 11		Age 12		Total	
	Boys	Girls	Boys	Girls	Boys	Girls
Leeds	—	—	508	532	508	532
Liverpool	101	186	242	498	343	684
East Anglia	199	255	294	279	493	534
Total	300	441	1044	1309	1344	1750

For the second part of the investigation 692 children aged 11 and 12 years were examined; the composition and locations of the sample are given in Table 2. 296 lived in Colchester where the water contains fluoride at varying levels but with a range of between approximately 1·2 and 2·0 p.p.m. (FORREST, BRANSBY and PARFITT, 1951)

TABLE 2. COMPOSITION OF THE GROUP EXAMINED FOR GINGIVITIS IN HIGH AND LOW FLUORIDE AREAS

	Norwich		Colchester		Total
	Age 11	Age 12	Age 11	Age 12	
Girls	113	96	43	101	353
Boys	85	102	45	107	339
Total	198	198	88	208	692

and 396 lived in Norwich where the fluoride content of the water is very low, less than 0·17 p.p.m. Dental mottling was more frequent in Colchester and the dental caries prevalence considerably higher in Norwich. The children were from secondary modern schools and the groups were comparable from a socio-economic point of view.

Standards of gingivitis

The difficulty of establishing standards for the recording of gingival conditions is well known. Methods used have included simple "present or absent" classifications (RUBRA, 1948); an arbitrary incidence and severity classification such as "absent, mild, moderate or severe" (MASSLER and SCHOUR, 1951); methods whereby recognized gingival conditions rate a predetermined numerical score, such as the PMA index introduced by MASSLER *et al.* (1950), or the modification of it suggested by PARFITT (1957); and a classification by RUSSELL (1956) which includes a wider range of periodontal conditions.

In the present investigation three standards were recorded, as follows:

1. *Good.* Healthy gingivae. The health line is well marked and stippling is evident. No evidence of inflammation.

2. *Poor.* Considerable inflammation of the gingivae with redness, tendency to bleed on pressure, engorged interdental papillae and gingival margins.

3. *Fair.* Any condition which does not fall into either of the two preceding categories.

Only the upper and lower labial segments were recorded as it has been shown by MASSLER *et al.* (1950) that these are a valid index of gingivitis experience of the entire mouth.

One of us (P.M.C.J.) habitually scores gingivae in PMA units giving each of six oral segments a PMA rating as classified by PARFITT (1957). It was found that category "Good" coincided with PMA total scores of 0–2 for the two anterior segments; "Fair" with PMA scores 2–4; and "Poor" with PMA scores of 5 or more. This implies that there is sometimes a very mild degree of inflammation present in cases classified here as normal, or without gingivitis. This difficulty has already been raised by MASSLER *et al.* (1950) who state that their category termed "mild"—"might be questioned by some who prefer to include such very mild inflammatory changes within the range of normality". In the present investigation cases showing these sub-clinical signs were placed in the lowest group.

Standards of dental cleanliness

MASSLER and SCHOUR (1951) rate dental cleanliness as very clean, clean, fair, poor and very poor. In the present investigation only three categories were used; as follows:

Good. The teeth are clean. There is no sign of food debris or materia alba when examined with an explorer.

Poor. The teeth are very dirty. There is considerable food debris of long standing, materia alba or heavy staining.

Fair. This class falls between the two preceding. There is some evidence of debris, but not to the degree recognized as poor.

Differences between examiners

It was hoped that by using broad divisions of diagnostic standards the difference between examiners would be minimized. To test the measure of agreement each of the four examiners examined the same group of 100 children aged 12 years and recorded gingival and dental cleanliness standards. The results are shown in Tables 3 and 4. From these it was evident that there was disagreement between examiners in their diagnosis and that this was of such an order as to make comparisons between Leeds, Liverpool and East Anglia invalid. Differences between sexes and age groups, and the relationship between gingivitis and dental cleanliness could still be studied in each area separately however, as these would be valid internal comparisons.

Only one examiner was concerned with the gingival classification of children in high and low fluoride areas so comparisons between these groups are permissible.

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Table 3. Standards of gingival condition recorded by four examiners in the same group of 100 boys aged 12 years

Examiner	Good	Fair	Poor
1	56	36	8
2	32	59	9
3	30	67	3
4	42	45	13

Table 4. Standards of dental cleanliness recorded by four examiners in the same group of 100 boys aged 12 years

Examiner	Good	Fair	Poor
1	54	35	11
2	48	38	14
3	42	56	2
4	61	32	7

RESULTS

Incidence of gingivitis and dental cleanliness

Tables 5 and 6 show the incidence of good, fair and poor gingival conditions in 11 and 12 year old children, boys and girls, in Leeds, Liverpool and East Anglia.

TABLE 5. 11 YEAR OLD CHILDREN. THE INCIDENCE OF GINGIVAL CONDITIONS IN 300 BOYS AND 441 GIRLS, EXPRESSED AS PERCENTAGES

Area	Good		Fair		Poor	
	Boys	Girls	Boys	Girls	Boys	Girls
Liverpool	31·68	49·46	44·55	36·02	23·76	14·52
East Anglia	43·22	30·20	40·20	52·16	16·58	17·65

TABLE 6. 12 YEAR OLD CHILDREN. THE INCIDENCE OF GINGIVAL CONDITIONS IN 1044 BOYS AND 1309 GIRLS, EXPRESSED AS PERCENTAGES

Area	Good		Fair		Poor	
	Boys	Girls	Boys	Girls	Boys	Girls
Leeds	61·61	70·68	26·57	21·62	11·81	7·71
Liverpool	29·75	50·41	48·76	37·55	21·49	12·05
East Anglia	31·63	48·75	44·56	39·07	23·81	12·19

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Tables 7 and 8 show the incidence of good, fair and poor standards of dental cleanliness in 11 and 12 year old children, boys and girls, in Leeds, Liverpool and East Anglia.

TABLE 7. 11 YEAR OLD CHILDREN. THE INCIDENCE OF STANDARDS OF DENTAL CLEANLINESS IN 300 BOYS AND 441 GIRLS, EXPRESSED AS PERCENTAGES

Area	Good		Fair		Poor	
	Boys	Girls	Boys	Girls	Boys	Girls
Liverpool	41·58	40·32	48·51	51·61	9·90	8·06
East Anglia	19·10	33·73	43·22	50·59	37·69	15·69

TABLE 8. 12 YEAR OLD CHILDREN. THE INCIDENCE OF STANDARDS OF DENTAL CLEANLINESS IN 1044 BOYS AND 1309 GIRLS, EXPRESSED AS PERCENTAGES

Area	Good		Fair		Poor	
	Boys	Girls	Boys	Girls	Boys	Girls
Leeds	37·01	64·85	48·62	30·83	14·37	4·32
Liverpool	28·93	51·20	55·79	41·77	15·29	7·03
East Anglia	19·73	41·22	39·80	41·94	40·48	16·85

To test the difference in standards of gingival health and dental cleanliness between boys and girls, and between 11 and 12 year old children, difference of proportion tests (HILL, 1955) were applied in turn to categories "Good" and "Poor" in the appropriate

age groups. Table 9 gives the data and significance levels of the difference between boys and girls. Table 10 gives the data and significance levels of the difference between 11 and 12 year old children.

The relationship between gingival state and dental cleanliness

Chi-squared tables were prepared relating dental cleanliness and gingival state in boys and girls of each age group in each area. All these were 3×3 tables except where the expected frequency in some cells was less than 5, and in these cases categories "Fair" and "Poor" were combined to allow a valid test. This occurred in four of the tables. Table 11 gives χ^2 values and the significance levels obtained from each.

Table 9. The difference between boys and girls in Good and Poor standards of gingival health and dental cleanliness

	Age	Locality	Totals		Standard	Boys		Girls		Significance of difference in percentage (%)
			Boys	Girls		No.	%	No.	%	
Gingival state	11	Liverpool	101	186	Good	32	31·68	92	49·46	1
					Poor	24	23·76	27	14·52	None
		East Anglia	199	255	Good	86	43·22	77	30·20	1
					Poor	33	16·58	45	17·65	None
	12	Leeds	508	532	Good	313	61·61	376	70·68	0·1
					Poor	60	11·81	41	7·71	5
		Liverpool	242	498	Good	72	29·75	251	50·41	0·1
					Poor	52	21·49	60	12·05	0·1
		East Anglia	294	279	Good	93	31·63	136	48·75	0·1
					Poor	70	23·81	34	12·19	0·1
State of dental cleanliness	11	Liverpool	101	186	Good	42	41·58	75	40·32	None
					Poor	10	9·90	15	8·06	None
		East Anglia	199	255	Good	38	19·10	86	33·73	0·1
					Poor	75	37·69	40	15·69	0·1
	12	Leeds	508	532	Good	188	37·01	345	64·85	0·1
					Poor	73	14·37	23	4·32	0·1
		Liverpool	242	498	Good	70	28·93	255	51·20	0·1
					Poor	37	15·29	35	7·03	0·1
		East Anglia	294	279	Good	58	19·73	115	41·22	0·1
					Poor	119	40·48	47	16·85	0·1

TABLE 10. THE DIFFERENCE BETWEEN 11 AND 12 YEAR OLD CHILDREN IN GOOD AND POOR STANDARDS OF GINGIVAL HEALTH AND DENTAL CLEANLINESS

	Sex	Locality	Totals		Standard	11 years old		12 years old		Significance of difference in percentage (%)
			11 yrs	12 yrs		No.	%	No.	%	
Gingival state	F	Liverpool	186	498	Good	92	49·46	251	50·41	None
					Poor	27	14·52	60	12·05	None
		East Anglia	255	279	Good	77	30·20	136	48·75	0·1
					Poor	45	17·65	34	12·19	None
	M	Liverpool	101	242	Good	32	31·68	72	29·75	None
					Poor	24	23·76	52	21·49	None
		East Anglia	199	294	Good	86	43·22	93	31·63	1
					Poor	33	16·58	70	23·81	5
State of dental cleanliness	F	Liverpool	186	498	Good	75	40·32	255	51·20	5
					Poor	15	8·06	35	7·03	None
		East Anglia	255	279	Good	86	33·73	115	41·22	None
					Poor	40	15·69	47	16·85	None
	M	Liverpool	101	242	Good	42	41·58	70	28·93	5
					Poor	10	9·90	37	15·29	None
		East Anglia	199	294	Good	38	19·10	58	19·73	None
					Poor	75	37·69	119	40·48	None

TABLE 11. THE RELATIONSHIP BETWEEN GINGIVAL STATE AND DENTAL CLEANLINESS IN 3094 CHILDREN

Age	Sex	No.	Area	χ^2	*P*
11	F	186	Liverpool	20	0·001
	M	101	Liverpool	12·6	0·001
	F	255	East Anglia	10·4	0·05
	M	199	East Anglia	9·9	0·05
12	F	532	Leeds	80	0·001
	M	508	Leeds	81	0·001
	F	498	Liverpool	70	0·001
	M	242	Liverpool	53	0·001
	F	279	East Anglia	11·6	0·05
	M	294	East Anglia	26	0·001
Total children		3094	All areas	433	0·001

Gingival health in high and low fluoride areas

Table 12 shows standards of "Good" and "Poor" gingival health occurring in Norwich and Colchester, together with the results of difference of proportion tests, as previously described.

Table 12. The difference between Colchester and Norwich children in Good and Poor standards of gingival health

Age	Sex	Totals		Standard	Norwich		Colchester		Significance of difference in percentage (%)
		Norwich	Colchester		No.	%	No.	%	
11	F	113	43	Good	26	23·01	18	41·86	5
				Poor	22	19·47	6	13·95	None
	M	85	45	Good	48	56·47	19	42·22	None
				Poor	9	10·59	8	17·78	None
12	F	96	101	Good	50	52·08	48	47·52	None
				Poor	10	10·42	15	14·85	None
	M	102	107	Good	26	25·49	49	45·79	0·1
				Poor	28	27·45	16	14·95	5

DISCUSSION

In every area, and in each sex and age group a clear relationship was found between standards of dental cleanliness and gingival standards. This is in agreement with established clinical impressions and with the work of Massler and Schour (1951) and Brown *et al.* (1954).

The reason for the dental cleanliness is not established in this investigation. In many cases it may be due to the successful use of a toothbrush; in others the individual muscle actions, the occlusion of the dental arches, or the nature of the diet consumed may be important factors. Correct toothbrushing and consequent removal of debris from the gingival margins would be expected, on the basis of this investigation, to improve the gingival state, although care must be taken in interpreting the results. Parfitt *et al.* (1958) found little improvement in the gingivae of schoolchildren following intensive dental health education but this may only demonstrate that health education, like any other form of advertising, must be continuous to be effective and even then carries no guarantee that the advice will be followed.

The comparisons between incidence of gingival standards and dental cleanliness in girls and boys aged 12 was clear cut, girls being better than boys, and boys worse than girls in all the comparisons. Similar uniformity was not found in the 11 year old children; in East Anglia the gingivae of 11 year old boys were better than 11 year old girls while in Liverpool the reverse was the case.

Study of the literature on the relative incidence of gingivitis between the sexes indicates that the difference may be associated with the age groups under consideration. MASSLER *et al.* (1950) found that girls had a higher incidence than boys in the age range 5–8 years, while in the 9–14 year group the incidence was higher in boys. STAHL and GOLDMAN (1953) found no difference in the incidence of gingivitis between boys and girls in the age group 5–13 years, but that boys aged 13–17 years had a significantly higher incidence than girls of the same age. PARFITT (1957) in a longitudinal study of English orphanage children showed that the incidence and severity of gingivitis reaches a maximum at 13½ years in boys, and at 11 years in girls. Before the age of 12 years the girls showed the higher incidence of gingivitis; at 12 years the position was reversed, boys showing the higher incidence.

All these studies suggest the same pattern of change between boys and girls although there are differences in assessment and in the age at which the change occurs. It might be that in the present study the change-over had already taken place among the 11 year old children of Liverpool but not yet in the 11 year old children of East Anglia. This may be supported by the fact that the general tendency for gingival health to improve with age in girls and deteriorate with age in boys was most marked in East Anglia.

Possible reasons for this dissimilar pattern can only be conjectured. It cannot be explained in terms of standards of dental cleanliness. Review of the time of year in which the respective children were examined does not suggest the possibility of any seasonal influence. If, as suggested by PARFITT (1957), the peaks in gingivitis incidence are associated with the onset of puberty it may be that ethnic variation accounts for the difference.

The second part of the investigation showed that there was no significant increase in the incidence and severity of gingivitis in children living in the high fluoride area. In fact, where significant differences occurred, children living in Colchester showed better gingival health. It is interesting to note that in Colchester, boys and girls of each age group had similar gingival conditions while in Norwich there was a marked tendency for girls to improve between the ages of 11 and 12 years and boys to deteriorate over this period.

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ERRATA

H. ROSENMAN, H. A. CHARIPPER and S. S. STAHL: The effects of stress on the submaxillary glands of young adult male rats. Selected histochemical observations

In this paper (*Arch. oral Biol.* **2**, 196–202) an error was made in the preparation of the accompanying plates in that both are presented upside-down and consequently incorrectly numbered. Therefore in Plate 1 the figure designated 1 is Fig. 6, the figure designated 2 is Fig. 5, etc., and similarly in Plate 2 the figure designated 7 is Fig. 10, etc. We regret the inconvenience this error may have caused the authors and our readers.

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CHARACTERIZATION OF SALIVARY COMPONENTS SEPARATED BY PAPER ELECTROPHORESIS

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Abstract—Using a standardized procedure of paper electrophoresis, seven fractions were consistently separated from parotid saliva. These components were eluted from the paper strips and analysed chemically and immunochemically. Serum proteins (albumin, α, β and γ-globulins) comprised 25 per cent of the total. The specific salivary proteins included amylase, two non-antigenic glycoproteins, and an unidentified antigenic cationic protein. The two glycoproteins (accounting for 35 per cent of the total protein) were cationic, contained hexoses, fucose and hexosamine in a ratio of 1:1 and little if any sialic acid.

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SEVERAL studies of saliva employing electrophoresis as a means of separating individual components have been reported and have indicated the complexity of this fluid (KINERSLY, 1953; KINERSLY and HOGBERG, 1955; DREVON and DONIKIAN, 1956; KINERSLY and LEITE, 1957; ZIPKIN, ADAMIK and SAROFF, 1957; KOSTLIN and RAUCH, 1957; PATTON and PIGMAN, 1957, 1958; FERGUSON, KRAHN and HILDES, 1958; HOERMAN, 1959; VERSCHURE, 1959; GABLE and EGGER, 1959; GELLER, HAMES and ROVELSTAD, 1959; GELLER and ROVELSTAD, 1959; WEINSTEIN, HESKINS and JACKSON, 1960). Of the several substances disclosed, only the enzyme, salivary amylase, could be identified with certainty (PATTON and PIGMAN, 1957). The results of the various electrophoresis studies were in fair agreement with respect to the distribution of components, but the variations indicated that there were many details which required clarification.

The data described below indicate that reproducible patterns may be obtained with a standardized procedure of paper electrophoresis. In addition, it has been possible to characterize the components thus disclosed by analysing samples eluted from the paper strips.

METHODS

Collection and preparation of saliva samples

Paraffin-stimulated whole saliva was collected in iced tubes containing a small amount of thymol. Extra-parotid saliva was secured by obstructing the orifices of Stensen's ducts with Lashley cups and collecting the fluid which pooled in the sublingual area with a saliva ejector connected to a vacuum pump through a collecting flask. In both cases, the samples were immediately centrifuged in the cold (4°C) for 1 hr at 1200 *g*, and the supernatant fluid was lyophilized without delay. Parotid saliva was obtained using Lashley cups as modified by Stephan. Stimulated submaxillary saliva was obtained by cannula as described by KERR (1955). These two

secretions were lyophilized directly since they were not contaminated with oral debris. The only exceptions to the above were samples which were to be analysed for total protein and protein-bound carbohydrate. In these cases phosphotungstic acid (*vide infra*) was employed as a precipitant and the appropriate analyses were made on the precipitates so obtained. For electrophoresis, the lyophilized powders were dissolved in a volume of 0·1 M NaCl such that the final protein concentration would be 4–6%. Before analysis, solutions were centrifuged for 45 min and then dialysed for 24 hr against the buffer used for electrophoresis.

Electrophoresis

The general procedure was that described by BLOCK, DURRUM and ZWEIG (1958). The Spinco Model R cell was used with S&S 2043A mgl paper. Two to four aliquots of 10 λl each were put on the strips with an applicator so as to provide samples of 1·2–1·5 mg protein per strip. Electrophoresis was carried out at 4°C for 4 or 16 hr at 10–15 mA per cell. Veronal buffer (pH 8·6, $\mu = 0{\cdot}05$) was ordinarily used, but other buffers were also tested. Paper strips were stained with bromphenol blue (BPB) to detect proteins, or with diphenylamine to localize carbohydrates.

Elution from strips

The procedure employed was similar to that of GOA (1955). After electrophoresis the paper strips were rapidly air-dried until they were only slightly damp. A thin segment was cut lengthwise from the centre of each strip, oven dried, and stained with BPB. These were employed as patterns for cutting the remainder of the strips into segments so as to separate the portions of the paper containing the individual components. By pooling such pieces from a number of strips (up to sixteen) quantities of the several substances sufficient for analysis were obtained by elution. The eluants employed were veronal buffer (for immunological tests), phosphate buffer pH 6·8 with 1 M NaCl (for amylase assay), or 0·01 M NaOH (for determination of protein, methyl pentose, or hexosamine). If hexose analysis was planned, the paper strips were washed with 0·1 N HCl and then with water prior to electrophoresis, and water was used for elution.

Chemical methods

Amylase was assayed with a soluble starch substrate as described by BERNFELD (1955).

Nitrogen was estimated with the Folin–Ciocalteu phenol reagent (KABAT and MAYER, 1948). The colorimetric standard contained 10γ of tyrosine nitrogen. Protein was calculated by multiplying the tyrosine N so determined by 6·25. Samples that had been dialysed to remove non-protein constituents were analysed directly. Protein was precipitated from undialysed samples with 5% phosphotungstic acid in 2 N HCl and the precipitate dissolved in 0·1 N NaOH for analysis.

Hexose was measured with the primary cysteine reaction (DISCHE, 1955, p. 338) and the anthrone method (SCOTT and MELVIN, 1953). Since qualitative analysis showed that the hexose consisted of galactose and mannose, a standard solution which

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contained equal amounts of these sugars was used. Small amounts of anthrone-positive substances remained on the paper strips after they were washed (HUFFMAN, 1955). It was therefore necessary, when analysing eluates after electrophoresis, to include blanks prepared from control strips. Careful filtration of the eluates was essential to remove shreds of cellulose. With the anthrone reaction, it was also necessary to correct the measured total values for the methylpentose (fucose) which is present.

Fucose was determined by the cysteine–H_2SO_4 method (DISCHE, 1955, p. 327).

For hexosamine, the Elson–Morgan reaction as described by SCHIFFMAN, HOWE and KABAT (1958) and by BOAS (1953) was used. The samples were hydrolysed in 2 N HCl for 4 hr in tightly stoppered 10 ml volumetric flasks and then evaporated to dryness in a vacuum desiccator over phosphorus pentoxide and sodium hydroxide. Each sample was dissolved in 4 ml of water and passed through a Dowex-50 column to remove non-hexosamine chromogens. The hexosamines were eluted with 2 N HCl from the resin. The alternative method, which uses isoamyl alcohol to eliminate the non-hexosamine chromogens (NEUHAUS and LETZRING, 1957), was also used in some instances. Both methods gave comparable results.

Immunochemical analysis

The veronal-buffer eluates were tested against anti-saliva sera, and against antisera to various serum proteins. Fractions were studied in Preer double-diffusion tubes (PREER, 1956) and by immunoelectrophoresis (SCHEIDEGGER, 1957). Details of the preparation of the antisera and immunological techniques are given elsewhere (ELLISON, MASHIMO and MANDEL, 1960).

RESULTS

Typical electrophoretic patterns obtained with whole saliva, parotid saliva and submaxillary saliva are illustrated in Fig. 1. These were obtained using veronal buffer pH 8·6, $\mu = 0{\cdot}05$. Electrophoresis was performed for 16 hr at 10–12 mA per cell. When a 4 hr electrophoresis period was employed, the rapidly migrating anionic components were sometimes more apparent, but the other substances were poorly resolved.

Six to eight fractions were usually detected in parotid saliva when the strips were stained with BPB and inspected visually. Readings with a photo-electric scanner showed six peaks, a shoulder at the origin due to material which did not migrate, and the presence of some protein between the fractions (Fig. 2a). The most rapidly migrating anionic substance frequently spread over a wide area, and since its concentration was low it was not appreciably stained with BPB (Fig. 2b). Analysis of eluates for protein and other tests (see below) indicated its presence. When the strips were stained with diphenylamine, carbohydrate was found mainly in two areas, corresponding to Bands 5 and 6, (*vide infra*).

Only four or sometimes five components could be found in whole saliva and in extra-parotid saliva. A large portion of the total protein remained at the origin and the mobile materials were not clearly resolved. These results were inferior to those

TABLE. I THE CHEMISTRY OF THE COMPONENTS OF PAROTID SALIVA SEPARATED BY PAPER ELECTROPHORESIS

(+)	1	2	sp	3	sp	4	sp	5	sp	6	sp	7	sp	(—)

Band	Protein (% of total)			Hexosamine[3] (% of total)		Fucose[4] (% of total)		Hexose[5] (% of total)	$\frac{\text{Hexose}(\gamma)}{\text{N}(\gamma)}$	$\frac{\text{Hexosamine}(\gamma)}{\text{N}(\gamma)}$	$\frac{\text{Fucose}(\gamma)}{\text{N}(\gamma)}$
	BPB[1]	Folin[2]									
	Ave.	Ave.	Range	Ave.	Range	Ave.	Range	Ave.			
1	1·0	9·4	5·0–11·7	0·9	0·7– 1·2	0·6	0– 1·9			0·087	
2	4·4	6·9	3·8– 9·5	1·3	0·9– 1·8	0·3	0– 2·3			0·056	
3		13·0	4·2–28·4	3·4	2·2– 4·8	0·3	0– 0·8			0·120	
4		11·5	4·8–18·1	6·2	5·7–10·1	1·1	0·8– 1·4			0·244	
3 + 4	57·1	25·9	21·6–36·3	11·5	9·3–14·3	1·3	1·0– 1·6			0·180	
5	7·3	12·1	11·1–13·9	14·0	10·0–17·2	17·3	15·6–17·2	26·5	3·1	0·438	0·456
6	17·9	23·5	14·8–36·5	57·5	53·5–62·4	67·6	59·0–73·5	41·3	2·8	0·881	0·889
7	1·3	9·3	6·2–15·3	3·4	2·8– 4·4	0·7	0– 1·9			0·119	
Spaces	11·0	17·1	11·9–24·1	12·3	4·5–18·9	10·9	6·7–13·6	34·1			

[1] Determined from densitometric measurements of strips stained with bromphenol blue.
[2] Tyrosine N measured by Folin–Ciocalteau method.
[3] Modified Elson–Morgan reaction.
[4] Cysteine reaction.
[5] Primary cysteine reaction and anthrone reaction.
sp Represents the unstained areas between the stained bands.

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obtained with parotid saliva, although one would have predicted that whole saliva should be more complex than the secretion from a single gland. This probably resulted from the continuing action of oral bacteria on whole saliva during collection. Experimentally, this was shown by comparing the patterns given when the whole saliva was collected in a tube containing parotid saliva and then processed, with those obtained with parotid saliva alone and with parotid saliva to which was added the supernatant fluid obtained after the centrifugation of whole saliva (Fig. 3). The first of these samples showed only the "whole saliva" pattern whereas the latter two were identical and showed the usual number of well separated fractions.

The paper electrophoretograms of the submaxillary saliva specimens collected by cannula were superior to those for whole saliva but failed to show as clear a pattern as did parotid saliva. It was apparent, however, that this secretion contained a rapidly moving anionic component which was not observed in parotid saliva (Fig. 1c).

The chemical and immunochemical studies of eluates from the paper strips made it possible to characterize and identify the substances which had been separated. Quantitative information concerning their distribution was also obtained. The results of these analyses are given in Tables 1 and 2.

Both BPB staining and analysis of the eluates for protein showed that usually there were two major constituents localized as Band 3 + 4 and Band 6. Occasionally Band 5 contained more protein than Band 6. The fraction of the total contained in Band 3 + 4 was lower when measured by chemical assay then when determined by staining (Table 1).

TABLE 2. ANALYSES OF PHOSPHOTUNGSTIC ACID-PRECIPITABLE PROTEINS IN PAROTID SALIVA

	Average (mg %)	Range (mg %)
Nitrogen	35·6	15·2–60·2
Hexosamine	11·3	7·4–22·8
Hexose*	44·0	13·0–86·0
Fucose	8·7	2·3–19·3

* Calculated as galactose + mannose.

Band 1 contained little carbohydrate and frequently was poorly resolved and spread over a large area, so that it was not well detected by staining. Immunological tests using antisera to human serum, to serum fractions, and to saliva indicated that it was probably serum albumin (Fig. 4).

Band 2 appeared also to be a serum protein since precipitation occurred when this material reacted with antiserum to human serum. It contained very little carbohydrate. Judging from its mobility, it was presumed to an α or β globulin.

Band 3 and Band 4 were considered as a unit because it was noted that although the proportion of the protein in these two bands was variable—sometimes more in Band 3 and on other occasions more in Band 4—the proportion of the total salivary

protein in the sum of the two bands was constant. When immunochemical tests were done, and checked by amylase activity measurements, it was determined that one of these bands contained amylase and the other contained γ-globulin, the amount of γ-globulin on the basis of nitrogen measurement always being less than the amount of amylase. The mobility of these two was similar and they were rarely completely separated into homogeneous fractions (Fig. 5). Under these circumstances it seemed futile to attempt to record them as separate fractions. The band contained a small amount of fucose and a larger amount of hexosamine.

Bands 5 and 6 were each composed of conjugated protein. Analysis of parotid saliva (Table 2) had shown that fucose (methyl pentose), hexose and hexosamine were present in significant quantity. Moreover, essentially all the carbohydrate was non-dialysable and precipitable by phosphotungstic acid, hence protein-bound. More than half of the hexose, hexosamine and fucose were associated with Band 6 and a lesser portion with Band 5. The ratio hexosamine : fucose was very close to unity in each band. The substance in Band 5 differed from that in Band 6 in that the ratios of hexosamine : N, hexose : N, and fucose : N were all lower in the former. Neither of these conjugated proteins was antigenic in the rabbit.

Band 7, the most rapidly migrating cationic component, contained an antigenic material whose identity remains in doubt (Fig. 6). Very little carbohydrate was associated with it.

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DISCUSSION

In appraising the usefulness of zone electrophoresis as a method for the analysis of saliva, it was chiefly important to determine that the substances disclosed were real entities, of uniform composition, and distinct from each other. Although the results obtained by other investigators had been reported as reproducible, there was sufficient variation in the patterns described to make comparison of data of doubtful value. Many of the apparent inconsistencies among these reports can be explained and resolved in view of the data presented here.

In order to obtain a well-resolved pattern, it was essential that the sample for analysis be essentially free of contamination by oral debris. This was clearly demonstrated by the superiority of the electrophoretograms of parotid and submaxillary salivas to those achieved with whole saliva or extra-parotid saliva collected by expectoration. For observing minor components, it was necessary to place a sufficient amount of protein on the paper strip. By concentrating saliva to contain 4–5 per cent protein, and putting 1·2–1·5 mg on each strip, clearcut results were obtained. With the application of too much protein, visualization of separation became difficult, as the stain spread over a broad area. The current needed for separation was about four times that which suffices for human serum.

Our findings with respect to the number and location of individual fractions are in general agreement with those of FERGUSON, KRAHN and HILDES (1958) and of KOSTLIN and RAUCH (1957). Comparison of free and paper electrophoresis is rendered difficult not only because of the inherent differences due to technique but also because of the lack of agreement in the results of boundary electrophoresis studies. Our

results compare closely to those reported by ZIPKIN, ADAMIK and SAROFF (1957), and to a lesser degree with those of PATTON and PIGMAN (1958) and GELLER, HAMES and ROVELSTAD (1959). PATTON and PIGMAN described several anionic components in parotid saliva with a higher mobility than serum albumin. These were not observed in our patterns nor by any other investigators. The other components they have described seem to have counterparts in those we found. Most workers, including ourselves, have found three cationic components, but GELLER, HAMES and ROVELSTAD (1959) observed only one. It seems quite possible, therefore, that the cationic material which they have labeled "lysozyme" was an unresolved mixture.

The limited studies which we have so far made of submaxillary saliva obtained by cannula are in overall agreement with the results reported by KOSTLIN and RAUCH (1957). The rapidly moving anionic component in submaxillary saliva and absent from parotid saliva may be a part of the submaxillary mucoid. GELLER, HAMES and ROVELSTAD (1959) noted such a fraction. On the other hand, PATTON and PIGMAN (1958) did not note such a material, although, as stated above, they described two rapidly migrating anions in parotid saliva.

In a number of studies, the separated fractions were assigned labels despite the absence of sufficient data to justify conclusions as to the identity of the substances. By analysing the materials eluted from the paper strips, it was possible to identify and characterize the components and to resolve some of the disparities between the results previously given.

The immunochemical tests made it possible to ascertain that γ-globulin was present as well as amylase, in Band 3 + 4. This had been implied in previous reports from the presence in saliva of substances with the qualitative characteristics of serum antibodies. If the gamma globulin in saliva came from serum, then other serum proteins should also be present. The immunological tests indicated that this was the case. Albumin was identified in Band 1 and α- or β-globulin in Band 2. These immunochemical criteria are rigorous and are generally accepted as a basis for identification. In addition, the protein nature of the substance in Band 7 may be inferred from the fact that it was antigenic in rabbits.

Band 3 + 4 appears to be comparable to the major component reported by all workers. The disparity between the proportion of the protein in this band as measured by scanning stained strips and nitrogen analysis of eluates probably was due to the fact that photoelectric scanning is unreliable when very dense (or very light) areas of staining are examined. The localization of amylase in this area is also in agreement with other reports (MCGEACHIN and LEWIS, 1959). The report of SCHNEYER (1956) and of FERGUSON, KRAHN and HILDES (1958) on the relation between amylase activity and protein concentration, as well as immunological data reported elsewhere (ELLISON, MASHIMO and MANDEL, 1960) indicate that the major portion of this band is amylase.

Positive reactions with carbohydrate stains, as employed by DREVON and DONIKIAN (1956) and by FERGUSON, KRAHN and HILDES (1958) and by us indicated the presence of glycoproteins. Chemical analysis of protein-bound carbohydrate in unfractionated saliva and of samples obtained by elution from strips substantiate this presumption. The consistent chemical differences between Bands 5 and 6, and the close agreement

of analyses of eluates from these bands obtained from numerous saliva samples, suggest that these are distinct and constant components of parotid saliva. A tentative composition as calculated from the data is that the glycoprotein in Band 5 contains 63 per cent protein, 5·5 per cent hexosamine, 5·5 per cent fucose and 25 per cent hexose, whereas that in Band 6 consists of 60 per cent protein, 10 per cent hexosamine, 10 per cent fucose and 20 per cent hexose. These substances could be classed as fucomucins similar in some respects to blood group substances rather than to the acid glycoproteins of submaxillary saliva and serum. Preliminary analyses of parotid saliva using several methods, including the thiobarbituric acid method (WARREN, 1959), indicated the presence of only 0·1 mg per cent of sialic acid, which is very much less than the amount which would be anticipated if the acid glycoprotein were present in any significant quantity (NISIZAWA and PIGMAN, 1959).

The import of the presence of these two glycoproteins in the parotid secretion cannot be ascertained on the basis of these data. Certainly, it is clear that despite the histological evidence which classifies the parotid gland as a serous gland, "mucoids" are nevertheless regularly secreted. It would be of interest to determine whether these substances could be involved in the formation of salivary calculus, particularly when the accretion is located on the buccal surfaces of the upper molar teeth.

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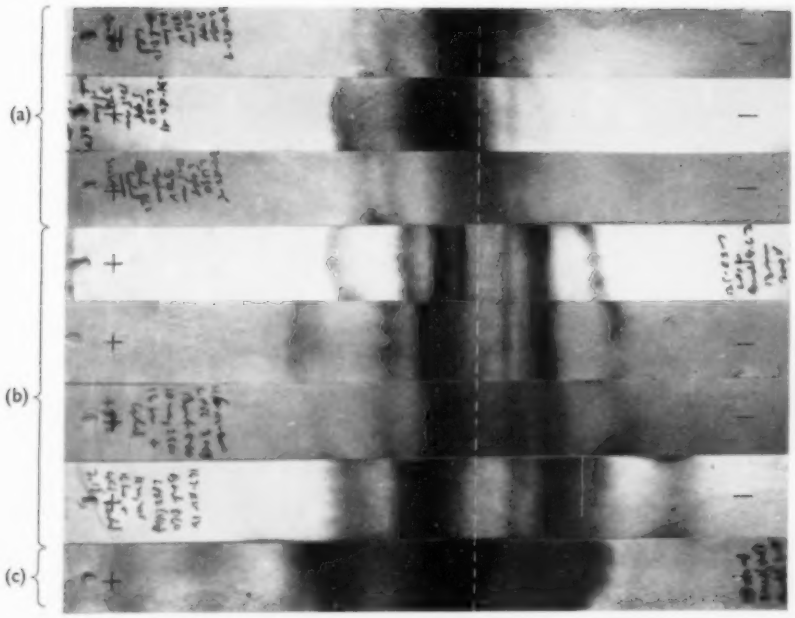

FIG. 1. Paper electrophoretograms stained with bromphenol blue. (a) Whole saliva. (b) Parotid saliva. (c) Submaxillary saliva.

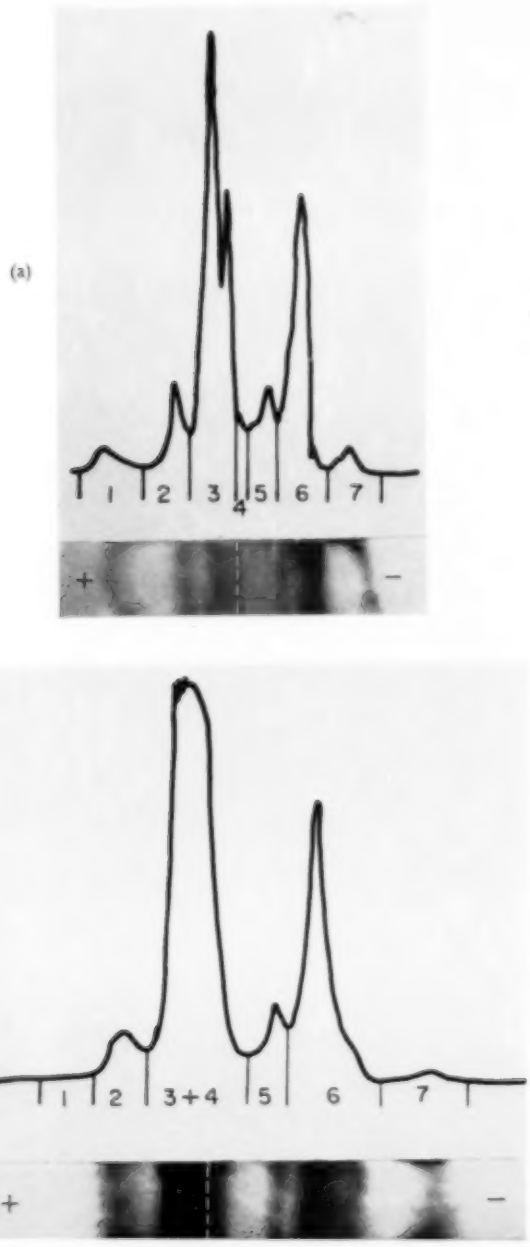

FIG. 2. (a) and (b). Paper electrophoretograms of parotid saliva stained with bromphenol blue and scanned photoelectrically with a recording densitometer.

PLATE 2

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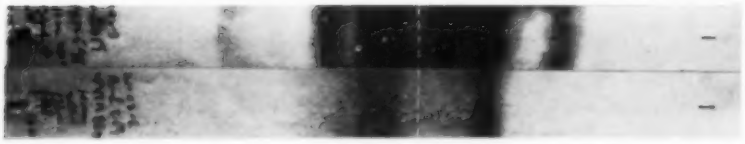

FIG. 3. Paper electrophoretograms stained with bromphenol blue. Upper strip—parotid saliva. Lower strip—whole saliva collected in a tube containing parotid saliva.

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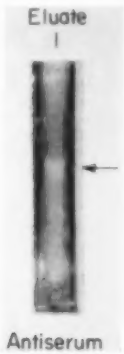

FIG. 4. Preer tube showing reaction between eluate from Band 1 and an antiserum to human serum albumin. Specific precipitate is marked with an arrow. (This convention is followed in succeeding photographs).

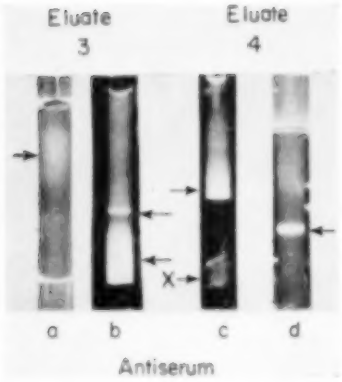

FIG. 5. Reactions between eluates from Band 3 and Band 4 in Preer tubes. (a) Anti-γ-globulin serum. (b) Anti-parotid saliva serum. (c) Anti-parotid, saliva serum (x indicates artifact). (d) Anti-parotid saliva serum absorbed with human serum.

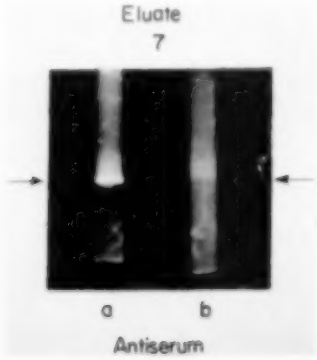

FIG. 6. Preer tubes. Reaction between eluate from Band 7 and antiserums. (a) Anti-parotid saliva serum. (b) Anti-parotid saliva serum absorbed with human serum.

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THE DEVELOPMENT *IN VIVO* AND *IN VITRO* OF THE LOWER INCISOR AND MOLARS OF THE MOUSE

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Abstract—The normal development is described of the lower incisor of the mouse from the eleventh day of foetal life until the sixth day after birth and the development of the first and second molars is briefly outlined.

Lower incisor and molar tooth germs were explanted from 13–18 day mouse foetuses and cultivated *in vitro*. They developed fairly normally until a layer of dentine had been laid down. Differentiation usually stopped at this stage, but in some explants a little enamel was formed.

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INTRODUCTION

MOLAR tooth germs from rat and rabbit foetuses have been cultivated *in vitro* by GLASSTONE (1936, 1938a, 1952) and from rat foetuses by LEFKOWITZ and his colleagues (LEFKOWITZ, MARDFIN and BODECKER, 1954; LEFKOWITZ and SWAYNE, 1956a,b). The development *in vitro* of whole incisor tooth germs does not appear to have been studied previously. The present paper describes the development in organ culture of the lower incisors and molars excised from foetal mice at various developmental stages. These tooth germs are somewhat smaller than those of the rat or rabbit, and are therefore in some ways better suited to growth *in vitro*. COHN (1957) states that "molar teeth in mice have attributes which make them more suitable for dental investigation than are rat molars, and yet very little attention has been given them in comparison with that given these teeth in the rat".

For comparison with the experimental material, an account is included of the normal development of tooth germs in the mouse. This is fairly detailed for the incisor as there does not appear to be a complete histological description in the literature. The development of the molars, however, has been described by GAUNT (1955, 1956) and COHN (1957), and is therefore mentioned only very briefly in this paper. The sequence of events that they describe agrees well with that to be reported here, but the timing is somewhat different in the three studies. MAHN (1890) also described the development of the mouse molar, but he studied rather few stages. More work has been done on the development of rat tooth germs (ADDISON and APPLETON, 1915; GLASSTONE, 1938a; MELLANBY, 1939; LEFKOWITZ, BODECKER and MARDFIN, 1953).

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MATERIALS AND METHODS

The mice used throughout this work were cross-bred, and derived from several general laboratory stocks. The ages of the foetuses were determined where possible by observation of the vaginal plug in the mother, and otherwise by the external appearance of the foetus (GRUNEBERG, 1943).

Normal development. Serial sections of developing tooth germs, some excised and some *in situ*, were cut. By this method their normal development was studied from the ninth day of gestation until 15 days after birth.

The explants. Lower incisor and molar tooth germs from foetuses ranging in age from the thirteenth to the eighteenth day of gestation (Stages 16–33) were cultivated *in vitro* for a maximum of 32 days, but most explants were fixed after 7–15 days, and a few after only 3–6 days in culture. Of the 300 tooth germs cultivated rather more were molars than incisors, because it was possible to cultivate both the first and second molars of the older foetuses, while it was often difficult to explant the incisors because shortly before birth they become encased in a tube of bone.

The tissue was immersed in Tyrode solution while it was dissected aseptically from the foetuses with specially sharpened cataract knives and fine sewing needles mounted in glass rods. Before explantation, most of the surrounding tissue of the dental sac was removed, but sometimes a small amount of the oral epithelium remained adherent to the tooth germs. Occasionally a first molar tooth germ was explanted with part of the dental lamina attached to its posterior edge, and during the period of cultivation the second molar began to develop; the two tooth germs were usually separated when the explant was transferred to fresh medium.

Method of cultivation. Most of the tooth germs were cultivated in watch glasses (FELL and ROBISON, 1929), but hanging-drop cultures were made of some of the younger ones. The medium consisted of equal parts of fowl plasma and chick embryo extract, the latter being prepared by extracting one part of a minced-up 10–13 day chick embryo with 2 parts of Tyrode solution. Eight to twelve drops of medium were used for the watch glass cultures, and two drops for the hanging drop preparations.

The cultures were incubated at 37–38°C. Every 2–3 days the explants were gently freed from the clot with a fine knife and needle, washed in Tyrode solution and transferred to fresh medium. At the end of the period of cultivation, they were removed from the clot and rinsed in Tyrode solution before being fixed.

Controls. Controls were prepared for each batch of cultures to show the stage of development at which the tissue was explanted. Usually the lower jaw, or one side of it, from one or two foetuses of each litter was fixed and serially sectioned for this purpose, but sometimes only isolated tooth germs were used as controls.

Histological treatment. Most of the controls and the material used for the study of the normal development of tooth germs were fixed in Zenker's fluid containing 3–5% acetic acid (according to the age of the tissue); Carnoy's or Bouin's fluid, or 10% neutral formol saline was occasionally used. Decalcification of the foetal tissue was unnecessary. The jaws and tooth germs of young mice more than 1 day old were decalcified in either formol nitric (3 parts 10% formalin and 1 part 10% nitric acid),

or in 10% formic acid in normal saline. Most of the cultivated tooth germs were fixed in 3% acetic Zenker's fluid.

The fixed tissue was embedded in paraffin wax and serial sections were cut in one of several planes. The whole jaws were usually cut coronally (transversely); longitudinal-vertical or transverse-vertical sections were cut of one side of the lower jaw, and of isolated tooth germs. Most of the sections were stained with Delafield's haematoxylin and Chromtrope 2R, and some with Heidenhain's azan stain; a few specimens were stained by von Kossa's silver method to show calcification.

Reconstructed models. Some tooth germs were reproduced as scale models by the wax plate technique, and more durable ones were made of methyl methacrylate.

Terminology. In describing the lower jaw and the tooth germs, the following terms will be used:

Proximally or posteriorly: towards the squamosal-dentary joint. Distally or anteriorly: towards the symphysis of the two sides of the jaw. Buccally: towards the cheeks. Labially: towards the lips. Lingually: towards the tongue. Medially: towards the mid-line.

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NORMAL DEVELOPMENT OF TOOTH GERMS *IN VIVO*

Stage of development

There was much variation in the stage of development between litters of apparently the same age, but it was possible to arrange the litters in a developmental series, which was divided into numbered stages. In assigning a stage to any litter, in addition to the tooth germs, other structures in the jaw were considered; these included Meckel's cartilage, the dentary and the salivary glands. The development of these structures is outside the scope of this paper, and therefore the characteristics of each stage cannot be described in detail. For purposes of comparison of this material with that of other workers, however, some of the more outstanding features that can be seen in the foetal jaw are summarized below. The gestation period is 20–21 days.

Stage 10 (10*th day*). The mandibular rudiments join in the mid-line to form an arch.

Stage 11 (11*th day*). The oral epithelium grows in to form a tooth band.

Stage 12 (11*th day*). The tongue is represented by a mushroom-shaped outgrowth of the lower jaw.

Stage 13 (12*th day*). Mesenchymal cells condense to form Meckel's cartilage.

Stage 16 (12*th*–13*th day*). All the red blood corpuscles are nucleated up to this stage. Hereafter the number of non-nucleated erythrocytes gradually increases until by Stage 26 only a few corpuscles contain nuclei, and by Stage 31 none is nucleated.

Stage 17 (12*th*–13*th day*). The central intrinsic muscles of the tongue, and the submandibular and sublingual salivary glands begin to develop. The incisor tooth germ starts to invaginate.

Stage 18 (12*th*–13*th day*). Rudimentary hair follicles are present on the lower surface of the jaw. The first molar is represented by a bud-shaped swelling.

Stage 21 (14*th*–15*th day*). The procartilaginous forerunners of Meckel's cartilage unite in the mid-line to form the rostral process. The mental foramen begins to develop.

Stage 22 (14*th*–15*th day*). Some of the osteoid tissue is calcified. The incisor tooth germ is bell-shaped.

Stage 23 (15*th*–16*th day*). Meckel's cartilage reaches its stage of maximum development. The plates of bone that give rise to the inner and outer alveolar walls and the coronoid process are present.

Stage 24 (15*th*–16*th day*). Secondary cartilages, that give rise to the condylar and angular cartilages, develop near the posterior edge of the lateral plate of bone. The incisor tooth germ is encased in a tube of bone. The first molar is bell-shaped, and shows the beginning of cusp formation; the dental lamina is extending backwards to form the second molar.

Stage 26 (15*th*–16*th day*). In the incisor tooth germ the cells of the internal enamel epithelium are elongating to become columnar pre-ameloblasts.

Stage 28 (15*th*–16*th day*). Meckel's cartilage begins to regress: in the region of the mental foramen, its cells are hypertrophic.

Stage 30 (16*th*–17*th day*). Meckel's cartilage has almost disappeared in the region of the mental foramen and has been replaced by bone.

Stage 31 (17*th*–18*th day*). In the incisor, the pre-ameloblasts have elongated to become ameloblasts; dentine is present along about half the length of the tooth.

Stage 34 (17*th*–18*th day*). Extravascular red blood corpuscles appear in the stellate reticulum of the first molar.

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Early development of tooth germs

The tooth germs arise from a tooth band, which is developed from three separate ingrowths of the oral epithelium into the mesenchyme of the young mandibular arch. The two proximal ingrowths, one on each side of the arch, give rise to the dental lamina of the molar tooth germs, and from the anterior ingrowth, in the mid-line of the arch, the two incisor tooth germs and also the lip furrow band, originate. In the mouse, the first signs of ingrowth of the oral epithelium are seen on the eleventh day *in utero*; the ingrowths for the molar teeth begin to develop before those for the incisors. In the early stages the incisor and molar regions are quite separate but the incisors gradually grow back until at birth they lie under the first molars, and later extend posteriorly to all the molar teeth.

Development of the lower incisor

The main features in the development of the incisor are summarized in Table 1.

The lip furrow band, which eventually opens to form the buccal sulcus, is closely associated with the development of the incisor tooth germs. On the twelfth day (Stage 13), the epithelial ingrowth begins to divide into a more medial tooth band (from which the incisor develops), and a more lateral lip furrow band (see Fig. 1). Both bands gradually grow deeper into the underlying mesoderm, but the tooth band always extends further in a proximal direction than the lip furrow band. By the

thirteenth day (Stage 17) (Fig. 2) the incisor tooth germ is present and is beginning to invaginate to form the enamel organ. Very soon capillaries grow into the space between the tooth band and the lip furrow band; this knot of capillaries is very prominent in the living 13 day foetus.

TABLE 1. THE APPROXIMATE AGE AND DEVELOPMENTAL STAGE AT WHICH VARIOUS PHASES IN THE DEVELOPMENT OF THE LOWER INCISOR AND MOLAR TOOTH GERMS OCCUR

	Age	Stage	Incisor	First molar	Second molar
Days of gestation	12	13	Tooth band separates from lip furrow band		
	12–13	14		Well defined d.l.	
	12–13	17	t.g. begins to invaginate (Fig. 2)		
	12–13	19		Bud-shaped swelling on d.l. (Fig. 6)	
	13–14	22	e.o. bell-shaped		
	14	22+		Invagination of t.g. begins; i.e.e. and e.e.e. are recognizable	
	14–15	23	e.o. begins to differentiate		
	15–16	24		e.o. bell-shaped; beginning of cusp formation indicated (Fig 7); columnar pa. present	d.l. present
	15–16	26	Columnar pa. present		
	16	28	od. present at anterior end (Fig. 4)		
	17	30			e.o. bell-shaped; cusp-formation indicated
	17	31	am. present; epithelial papillae begin to develop; dentine present along about half the length of the t.g.		
	17	32	Calcification begins in dentine	Shown in Fig. 8	Shown in Fig. 8

TABLE 1 (*contd.*). THE APPROXIMATE AGE AND DEVELOPMENTAL STAGE AT WHICH VARIOUS PHASES IN THE DEVELOPMENT OF THE LOWER INCISOR AND MOLAR TOOTH GERMS OCCUR

	Age	Stage	Incisor	First molar	Second molar
	Shortly before birth	34	Development of enamel begins	Cusps project far into e.o. obliterating s.r. Numerous extravascular r.b.c. present in s.r.	
	Birth			od. develop; dentinogenesis begins; pa. stop dividing and elongate to form am.	r.b.c. present in s.r.; pa. have formed
Days after birth	1			Vascularization of s.r. reaches its peak; the cells of the e.e.e. are flatter and between them r.b.c. and connective tissue penetrate into the s.r. (Fig. 9)	od. present
	2			r.b.c. much less numerous in s.r., which has lost its characteristic structure and consists of a rather diffuse type of connective tissue; the crown is covered by a layer of dentine; amelogenesis begins	s.r. has been obliterated over cusps and a thin layer of dentine is present
	4			e.e.e. merges with s.r. and ceases to exist as a distinct layer; a layer of enamel is present	Enamel present
	6		Enamel is calcified		
	15			am. are very short with peripherally-placed nuclei; remains of e.o. are probably represented by stratified epithelium or cuboidal epithelium with finger-like projections	

am. ameloblasts; d.l. dental lamina; e.e.e. external enamel epithelium; e.o. enamel organ; i.e.e. internal enamel epithelium; od. odontoblasts; pa. pre-ameloblasts; r.b.c. red blood corpuscles; s.r. stellate reticulum; t.g. tooth germ.

By the thirteenth or fourteenth day (Stage 22), the tooth germ consists of a bell-shaped ectodermal enamel organ enclosing mesenchymal cells, which have condensed to form the dentine papilla. The lower or labial surface of the bell grows more extensively in a proximal direction, and develops further than the upper or lingual one (Fig. 3). Later, the whole tooth germ becomes flattened in a labio-lingual direction, to produce the characteristic spatulate form of the rodent incisor.

By the fourteenth–fifteenth day (Stage 23), the enamel organ has begun to differentiate and three layers can be distinguished in it. The outer layer consists of cuboidal cells and forms the outer enamel epithelium, the inner layer or inner enamel epithelium is composed of slightly more columnar cells, and between the two is a mass of relatively undifferentiated epithelial cells, the future enamel pulp (Fig. 3).

Later on the fifteenth day (Stage 26) the cells of the inner enamel epithelium elongate slightly to become columnar pre-ameloblasts; their nuclei are oval and centrally placed. At this stage these cells are of approximately equal length on the labial and lingual sides of the tooth germ. While this condition still obtains, the peripheral cells on the labial side of the anterior part of the dentine papilla differentiate into odontoblasts. This differentiation gradually spreads proximally down the labial side of the papilla, and also laterally and medially round to the lingual side. Fig. 4 shows a longitudinal section of an incisor at Stage 28. By this stage a well-marked concavity has appeared at the anterior end (future incisal edge) of the tooth germ.

On the seventeenth day (Stage 31), odontoblasts are present all round the anterior end of the dentine papilla, and on the labial side the pre-ameloblasts have further elongated to become ameloblasts, with peripherally placed nuclei; the stratum intermedium, a layer of flattened or somewhat rounded cells adjacent to the inner enamel epithelium, has differentiated from the inner part of the enamel pulp. The stratum intermedium is present all round the anterior part of the tooth germ; labially it overlies ameloblasts and lingually pre-ameloblasts. The enamel pulp has also differentiated into the stellate reticulum.

There is active cell division in the internal enamel epithelium both before and after its cells elongate to become pre-ameloblasts. When the pre-ameloblasts of the labial side elongate to become ameloblasts with peripherally-placed nuclei, they stop dividing, and at about the same time, cell division appears to cease in the pre-ameloblasts at the anterior end of the lingual side. It is at this stage (31) that the pre-ameloblasts of the lingual side reach their greatest length, which is only a little longer than when they first become columnar in Stage 26. In later stages these cells diminish in length, becoming first cuboidal and then flattened. They tend gradually to lose their identity and to merge with the surrounding connective tissue cells, but even in a 15-day old mouse traces of the lingual side of the enamel organ can be distinguished. ADDISON and APPLETON (1915) and MELLANBY (1939) have observed a similar regression of the lingual pre-ameloblasts in the rat.

Apart from the ameloblasts, the enamel organ of the incisor tooth is developed to only a very limited extent compared with that of the molars. In the incisors, the stellate reticulum is very thin except at the anterior end of the tooth, and the stratum intermedium and outer enamel epithelium are distinct for only a short period. The enamel organ is less well developed on the lingual than on the labial side. About Stage 31, the outline of the outer enamel epithelium at the anterior end on the labial side becomes wavy, and epithelial papillae consisting of the outer enamel epithelium and part of the stellate reticulum, develop (Fig. 5). Capillaries from the surrounding mesoderm grow into the indentations between the papillae. At a slightly earlier stage capillaries are frequently seen close to and parallel with the outer surface of the external epithelium.

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Very soon after some ameloblasts have developed (sixteenth–seventeenth day *in utero*), dentine is laid down by the odontoblasts; dentinogenesis begins on the labial side of the tooth germ, near its incisal edge, and by Stage 31 (seventeenth day), dentine is present along about half the length of the incisor. Dentine formation spreads rapidly to the lingual side of the tooth germ, and at the anterior end of the dentine papilla (or dental pulp, as it can now be called), some "irregular dentine" is laid down in the form of a cap (Fig. 5). This dentine does not appear to contain tubules, and it lies in amongst the cells of the pulp. By the second day after birth the anterior end of the dental pulp is filled with a solid block of "irregular dentine" in which are embedded odontoblasts and some pulp cells. The "irregular dentine" adjoins the normal tubular dentine. Calcification begins in both at about the eighteenth day *in utero*, very soon after they are laid down.

The layer of dentine gradually increases in thickness all round the developing tooth, but it is always thicker on the labial than on the lingual side. The apical (posterior) end of the tooth acts as a formative zone and its cells divide actively, contributing to the backward elongation of the tooth which is of persistent growth. Differentiation of the enamel organ and of the odontoblasts proceeds in a distal direction. The odontoblasts appear to migrate towards the incisal edge of the tooth (SCHOUR and STEADMAN, 1935), becoming more differentiated as they go, so that by the time they reach the posterior edge of the dentine cap, they are fully functional and begin to lay down dentine. Thus the layer of dentine increases in length.

Development of enamel begins just before birth on the labial side of the tooth germ near its incisal edge, and deposition progresses in a proximal direction. Enamel is never found at any stage on the anterior extremity of the labial side, on the incisal edge or anywhere on the lingual side of the tooth. By the fourth day after birth, the labial root tip of the incisor lies behind the molar region, and enamel is present as far back as that part of the incisor that underlies the second molar. At this stage, the enamel is probably not calcified and prisms are visible in it (MELLANBY, 1939), but it appears to be calcified by the sixth day, by which time its prismatic structure is no longer visible in undecalcified material. Between 2 and 4 days after birth, the anterior tip of the incisor is pushed into the epithelial stalk (the remains of the dental lamina) which connects the enamel organ with the oral epithelium. The epithelial stalk thickens to form a cap over the dentine which ceases to be surrounded by cells of the inner enamel epithelium. ADDISON and APPLETON (1915) made similar observations in the rat. By the fifteenth day after birth the tip of the incisor has just broken through the overlying epithelial tissue into the mouth.

The early development of "irregular dentine" is interesting. It seems to have similarities with both osteodentine and secondary dentine as described by TOMES (1923), but both these tissues usually develop later in life in response to injury. TOMES states, however, that "the axial portions of many teeth of persistent growth, for some little distance below the working surfaces, become filled by a calcification which has much in common with the secondary dentines—that is to say, it is of coarser and less definite structure than the peripheral parts of the same tooth". He goes on to say that: "In some Rodents the final closure of the axial tract takes place almost by a continuance

of the formation of normal fine-tubed dentine with very little secondary dentine of different structure, while in others there is a large area of dentine with vascular tracts in it". The mouse probably belongs to the second group. The early appearance of the irregular dentine, however, suggests that it is part of the process of normal development, and not the result of injury, as implied in the term "secondary dentine".

Development of the first and second lower molars

Table 1 shows the approximate age and developmental stage of the embryo at which various phases in the development of the molar tooth germs occur. The timing of events is of interest for comparison with the tooth germs cultivated *in vitro*.

DEVELOPMENT OF TOOTH GERMS *IN VITRO*

Preliminary experiments

The culture method used throughout most of this work, and already described, was adopted on the result of some preliminary experiments.

(i) Watch glass and hanging drop cultures were compared. The tooth germs used in this experiment were explanted from 13 day (Stage 19) and 15 day (Stage 30) foetuses, and were of such a size that they grew equally well in either vessel; on the basis of this result it was decided to use watch glasses as these were more convenient. When in a later experiment, however, an attempt was being made to cultivate some tooth germs from younger foetuses (Stage 17), it was found that hanging drop cultures were much more satisfactory for these very small explants.

(ii) Tyrode solution contains only 0·1% glucose. In two experiments the embryo extract was made with Tyrode solution to which 2% glucose and 0·2% calcium glycerophosphate had been added, but this was not found to have a beneficial effect. A thin shell of calcified material developed round the outside of many of the tooth germs, and the cells in the middle became necrotic.

(iii) GLASSTONE (1938a) obtained better results with rat and rabbit tooth germs by transferring them to fresh medium every 4–6 days rather than every 72 hr. The mouse tooth germs developed much more normally, particularly in shape, when transferred every 2 days; this was compared with transfer every 4 days.

The lower incisor

The development *in vitro* of incisor tooth germs isolated between the thirteenth and seventeenth days *in utero* (Stages 16 and 31) was studied. After Stage 31, the tooth germs become enclosed in a tube of bone from which they cannot be removed without stripping off part of the enamel organ.

At Stage 16, the incisor rudiments consist of epithelial thickenings which have not yet invaginated. These rudiments, together with a little underlying mesenchyme, were isolated and cultivated in watch glasses, but all the explants had degenerated by the time they were fixed after 4–12 days *in vitro*.

Many of the tooth germs explanted at Stage 17 (thirteenth day) also degenerated, but a few incisors continued to develop in hanging drops. When explanted, invagina-

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tion of the enamel organ had only just begun (Fig. 2). After 7 days *in vitro* pre-ameloblasts and odontoblasts had differentiated, there was a thin layer of dentine at the anterior end of the tooth germ, and in the pulp "irregular dentine" was beginning to form. After 11 days, ameloblasts were present in the anterior part of the tooth, although the pre-ameloblasts were still dividing near the posterior end; a thick layer of tubular dentine had formed down the labial side, (Fig. 10), and the anterior part of the pulp was filled with "irregular dentine".

By Stage 19 (thirteenth–fourteenth day *in utero*) the survival rate of the cultures was higher and, out of fifteen explants, five degenerated and six formed recognizable tooth germs. Of the latter, three were incisors which were cultivated for 10 days and all developed dentine; the other three were molars. The remaining four explants, which consisted mainly of connective tissue and epithelial ingrowths, contained many dividing cells and had the appearance of early tooth germs, but after 10 days *in vitro* they had not developed a typical stellate reticulum or formed any dentine. At this stage no difference could be seen between development in hanging drops and that in watch glasses.

Incisor tooth germs isolated between the fifteenth and seventeenth days (Stages 23 and 31) usually developed well *in vitro*. They enlarged considerably and sometimes maintained their characteristic shape, although frequently they became very distorted, probably because of damage to the outer layers of the tooth germ; the enamel organ was easily detached accidentally from the dentine of the anterior end during subcultivation. Histological differentiation, though slower than *in vivo*, proceeded fairly normally until quite a thick layer of dentine had been laid down (Fig. 11). Here differentiation *in vitro* frequently stopped, although some explants remained healthy for periods of up to 10 days after this stage had been reached. Some tooth germs developed to a more advanced stage in which a little enamel was produced, but this was not a constant feature and its occurrence will be described later.

Only a few explants were fixed after less than 10 days' cultivation, but these suggested that the early histological development of the incisor tooth germ *in vitro* was almost normal. A four-layered enamel organ developed in which the stellate reticulum was often very thin; its posterior formative end had an almost typical appearance, with many dividing cells. The cells of the internal enamel epithelium elongated to become pre-ameloblasts which also divided quite actively in culture, until on the labial side they differentiated into ameloblasts. Just before this happened, odontoblasts developed and began to lay down dentine, and "irregular dentine" appeared in the anterior end of the dental pulp; the enamel organ on the lingual side of the tooth was very thin.

About the same degree of development could be attained by all cultures of incisor tooth germs regardless of whether they were explanted at Stage 17 or Stage 30. The maximum amount of development was therefore achieved in tooth germs explanted at Stage 17 (about the thirteenth day); in them predentine was laid down by the seventh day *in vitro* and by the tenth day a layer of dentine had been formed comparable in thickness to that found in a normal 19 day foetus, i.e. 10 days' development *in vitro* was equivalent to about 6 days *in vivo*. Incisors explanted at Stage 24 (fifteenth day) appeared to develop slightly faster than this and achieved the equivalent of 4 days

of *in vivo* development in 5 days *in vitro*. During this period of active development *in vitro* the tooth germs elongated considerably and the characteristic concavity usually appeared in the incisal edge. Measurements were not made during cultivation, but a Stage 28 tooth germ is about 1·5 mm in length, and after 13 days *in vitro* such an explant measured about 3 mm.

Some cultures, having laid down a layer of dentine, began to degenerate. Degeneration usually began after 10–20 days *in vitro*, but the length of time for which an explant remained healthy varied very much in different batches of cultures, and seemed to have no relationship with the stage at which the tissue was explanted.

The lower molars

The development of the molar tooth germs *in vitro* was studied in rudiments explanted between the thirteenth and eighteenth days of gestation (Stages 16–34).

At Stage 16, the first molars are represented by thickenings in the oral epithelium. These thickenings were explanted with the mesenchyme immediately underlying them, but such rudiments degenerated when cultivated in watch glasses. One molar tooth explanted at Stage 17 developed quite well in a hanging drop, but it was unfortunately lost after fixation, so nothing is known of its histology. Neither molars nor incisors developed well in watch glasses at this stage.

At Stage 19 (thirteenth–fourteenth day *in utero*) (Fig. 6) the molar tooth germ consists of a bud-shaped thickening of the dental lamina round which mesenchymal cells are condensing. After 7 days *in vitro* a bell-shaped tooth germ had developed in which the cells of the internal epithelium were still cuboidal, and there was only a very slight indication of cusp formation; after 10 days pre-ameloblasts had developed, the cusps were much further advanced, and the stellate reticulum was well differentiated.

First molar tooth germs explanted between the fifteenth and eighteenth days of gestation (Stages 23–34) reached a more advanced stage of differentiation *in vitro* than when explanted at Stage 19, and very few rudiments degenerated. The tooth germs enlarged considerably (Fig. 12), and dentine was almost always laid down after a sufficiently long period of cultivation (Figs. 13 and 14). In a few explants, a little enamel was also formed.

The second molar is represented at Stage 23 by a small thickening of the dental lamina just behind the first molar. It was not observed in the living jaw, but must have been left attached to two first molar tooth germs explanted at this stage. After 15–17 days *in vitro*, an advanced bell-shaped second molar tooth germ had developed in which the stellate reticulum was present, and the cells of the inner enamel epithelium had just begun to elongate to form pre-ameloblasts. Second molar tooth germs, which developed in explants containing first molars excised at Stage 24 (fifteenth–sixteenth day *in utero*) formed a thick layer of dentine after 14–17 days *in vitro*, and in one upper molar a very small amount of enamel was laid down. The second molars developed more normally in shape if they were separated from the first after 4–6 days *in vitro*, by which time the two had usually become quite distinct. In Stage 30 (seventeenth day *in utero*) the second molar tooth germ was quite obvious in the living jaw and could be explanted alone, but such explants always degenerated and it was not

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until Stage 33 (seventeenth–eighteenth day *in utero*) that the second molar could be isolated at the time of explantation and continue to develop. (A second molar at Stage 32 is shown in Fig. 8.)

As *in vivo*, the first and second molar tooth germs developed more slowly *in vitro* than the incisors; when a layer of dentine had been formed over the surface of the crown of the molars, little further differentiation took place, except in the few explants in which a little enamel was laid down (see below). When the first and second molars from any foetus were explanted at the same time, the second molar was less advanced than the first at the beginning of the cultivation period, but it continued to develop after dentine had been laid down in the first molar; thus after about 12–15 days *in vitro* both were covered by a layer of dentine and were considered to have reached the stage of "maximum development". This stage is shown in Figs. 13 and 14 in first molar tooth germs explanted at Stages 28 and 31, respectively (Figs. 15 and 16).

First molar tooth germs explanted at Stage 23 (about the fifteenth day) achieved the stage of "maximum development" in 13 days; this stage would have been reached *in vivo* by the second day after birth, and therefore 1 day's development *in vitro* is equivalent to about 0·62 day *in vivo* (based on a 21-day gestation period). A similar calculation has been made for several batches of cultures explanted at different stages, and the results are given in Table 2 for the explant that reached the stage of "maximum development" in the shortest time. This stage may sometimes have been reached earlier than would appear from the table, but explants were seldom fixed at daily intervals; e.g. in Stage 31, explants were fixed on the eighth and fourteenth days of cultivation; by the eighth day "maximum development" had not been achieved, so it was not seen until the fourteenth day, though it probably occurred sooner. In most experiments, however, explants were fixed more frequently than in the example given above.

TABLE 2. THE TIME TAKEN BY FIRST MOLAR TOOTH GERMS OF THE DEVELOPMENTAL STAGE SHOWN, TO LAY DOWN A LAYER OF DENTINE *in vitro* AND *in vivo*

Stage when explanted	Approximate age when explanted (days)	No. of days *in vitro* to reach "maximum development"	No. of days *in vivo* to reach equivalent stage	No. of days *in vivo* equivalent to 1 day *in vitro*
23	15	13	8	0·62
24	15	12	8	0·66
24	15	11	8	0·73
28	16	13	7	0·54
30	17	11	6	0·55
30	17	9	6	0·67
31	17	14	6	0·43
33	18	8	5	0·62

Table 2 shows that the rate of development *in vitro* of the first molar tooth germ did not vary with the stage at which the tissue was explanted but, as with the incisors, more development was achieved by the tooth germs explanted at the earlier stages.

The second molar tooth germ was always much smaller than the first and reached its "maximum development" about 2 days after the first molar of the same foetus.

The histological development *in vitro* of the two molar teeth was very similar to that of the late foetal and early postnatal stages *in vivo*. When explanted at Stage 24, the first molar consisted of a dentine papilla and a four-layered bell-shaped enamel organ in which cusp formation was just beginning (Fig. 7), while the second molar was represented by a bud-shaped thickening of the dental lamina. The latter proceeded to invaginate and a bell-shaped enamel organ was thus formed. The development of pre-ameloblasts and of cusps advanced rapidly in both tooth germs; a layer of predentine was formed, followed by differentiation of odontoblasts, formation of a layer of dentine, and finally by the transformation of pre-ameloblasts into ameloblasts. "Maximum development" was reached by the first molar after 12 days' cultivation, and by the second molar after 14 days. The relative rates of development *in vitro* of the two tooth germs were shown when they were explanted together at Stage 30. After 5 days cusps were well developed in both; in the first molar the pre-ameloblasts were actively dividing and odontoblasts were just beginning to differentiate, while in the second molar the pre-ameloblasts were quite short and probably newly formed, and no odontoblasts were present. After 7 days' cultivation, in the first molar a thin layer of dentine had formed over the tips and on the sides of the cusps, and ameloblasts were differentiating; in the second molar the pre-ameloblasts were dividing, odontoblasts were differentiating and a layer of predentine was present. By the ninth day, there was quite a thick layer of dentine over the first molar, but none on the second. No second molar was fixed between the ninth and fourteenth days, but by the latter stage, a thick layer of dentine was present.

During cultivation most of the molar tooth germs increased considerably in size, although they did not enlarge as much as they would have done *in vivo* (Fig. 12). Some explants, probably damaged during dissection, degenerated without enlarging, and a few others which developed histologically in a normal way did not increase in size, and had the appearance of "miniature" tooth germs.

Part of the oral epithelium was sometimes left attached to the tooth germ and *in vitro* this formed a keratinized cyst (Fig. 13) which if large was generally removed when the tissue was transferred to fresh medium, as it was found that such cysts distorted the shape of the tooth.

The enamel organ of the molar teeth (Figs. 13 and 14) developed much further than that of the incisors (Fig. 11) and the ameloblasts showed no signs of regression even after 21 days' cultivation. The stellate reticulum was not invaded *in vitro* by connective tissue cells and did not degenerate; in first molars explanted at Stages 33 and 34, it already contained red blood corpuscles and a little connective tissue, which did not proliferate to any extent during cultivation. The red blood cells appeared to collect together in large groups near the edge of the explant (Fig. 14); they could still be seen quite clearly after 15 days *in vitro*. The outer enamel epithelium was poorly developed (Figs. 13 and 14); it was distinct in the early stages of cultivation but seemed to disappear when the stellate reticulum developed. Fully developed ameloblasts, though present on the sides of the cusps, were absent over their tips (Fig. 14).

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In some explants dentine with a rather abnormal structure was formed in addition to the layer of tubular dentine found. When this abnormal dentine was present in the cusps it often resembled the "irregular dentine" seen in incisors *in vivo*. At the sides of the molar tooth germs, sometimes odontoblasts which had laid down normal dentine peripherally seemed also to develop dentine around themselves, and nodules of tissue with a dentine-like appearance were present between the pulp and the odontoblasts. A third type of abnormal dentine was the "scar tissue" (an irregular mass of dentine) reported by GLASSTONE (1936); it formed at the edge of both molar and incisor tooth germs where the ameloblasts had been badly damaged or removed, after odontoblasts had differentiated.

Enamel

After a layer of dentine had been laid down, a small amount of enamel appeared in some cultures. In sections stained with haematoxylin and chromotrope, it was much more purple than the pink dentine. It varied greatly in both appearance and amount in different cultures. In some, it seemed to have very little structure, while in others it had a prismatic appearance, or consisted of large globules. Some cultures contained only a few droplets of material which stained the same colour as the enamel just described. Figs. 17, 18, 19 and 20 show several types of enamel which developed during cultivation *in vitro*. For comparison, enamel of a normal 4 day old mouse is shown in Fig. 21.

In a series of 219 isolated tooth germs, sixty developed some enamel-like substance, i.e. 23 per cent. Table 3 shows how this was distributed between the incisors and the

TABLE 3. THE OCCURRENCE OF ENAMEL-LIKE SUBSTANCE IN ISOLATED TOOTH GERMS CULTIVATED *in vitro*

Type of tooth germ	Number cultivated	Number in which enamel developed	% Occurrence
Incisor	83	34	41
First molar	106	23	22
Second molar	30	3	10
Total	219	60	23

first and second molars. In the incisors, the enamel always occurred on the labial side of the tooth, and in molars it was frequently found on the outer (peripheral) side of the cusps, near their tips (Fig. 20).

The stages at which the tooth germs were explanted did not seem to affect the development of enamel. It was produced by incisors explanted between Stages 17 and 31, by first molars from Stages 23 to 34, and by second molars explanted at Stages 24 and 30. Table 4 indicates how soon enamel developed in the different tooth germs explanted at the stages given. As enamel could not be seen with certainty in the living cultures, the shortest period of cultivation at the end of which enamel was present is recorded.

No factors were discovered which influenced the development of enamel *in vitro* and its formation was quite unpredictable. All the explants in each batch of cultures were taken from members of the same litter, and it was usually found that in a given batch either none of the explants produced enamel, or quite a high percentage did so, after a sufficiently long period of cultivation. No enamel was produced in six batches of cultures taken from foetuses between Stages 19 and 34; on the other hand the sixty tooth germs, in which enamel has already been reported, came from nine separate batches of cultures in which a total of 177 tooth germs were explanted between Stages 17 and 34, i.e. 45 per cent of these tooth germs produced enamel.

TABLE 4. SHORTEST PERIOD OF CULTIVATION AT THE END OF WHICH ENAMEL WAS PRESENT IN TOOTH GERMS EXPLANTED AT THE STAGES SHOWN

Tooth germ	Stage when explanted	No. of days *in vitro*
Incisor	17	11
Incisor	23	13
First molar	23	13
Incisor	24	9
First molar	24	11
Second molar	24	17
Incisor	28	9
First molar	28	10
Incisor	30	7
First molar	30	11
Second molar	30	14
Incisor	31	14
First molar	31	21
First molar	34	16

DISCUSSION

The development *in vitro* of tooth germs of the mouse has confirmed the observations of earlier workers that the teeth of many foetal and young mammals are able to continue their development in a remarkably normal way, when transplanted either as grafts or into organ cultures (GLASSTONE, 1954). Grafted tissue generally reaches a more advanced stage of differentiation than that cultivated *in vitro*, but in some respects the former is not so useful experimentally because the tissue is less completely isolated, and its new environment cannot be so well controlled as in culture. GLASSTONE (1938a, 1954) has shown how much further tooth germs advance developmentally when grafted on to the chick chorio-allantoic membrane, than when cultivated *in vitro*. A thick layer of enamel, such as was never seen in the cultures, developed in the grafts and was attributed to vascularization. FLEMING (1952) said that a good blood supply was necessary for amelogenesis in grafted mouse tooth germs.

It is probably not vascularization itself which is essential for enamel formation, but an adequate supply to the stratum intermedium and the ameloblasts of the materials necessary for amelogenesis. Support for this view can be found (i) in the lack of vascularization *in vivo* of the enamel organ of the mouse incisor, but in the presence on its labial side of epithelial papillae between which, in the mesenchyme, are capillaries and (ii) in the development of a little enamel in mouse tooth germs cultivated *in vitro*. More enamel seems to have formed in these cultures than in those made by earlier workers of the developing teeth of rats and rabbits. The only published photomicrograph of enamel developed *in vitro* appears to be one by GLASSTONE (1938a) of a rabbit molar in which, at high magnification (×1400) a very small amount of enamel can be seen. It seems likely that enamel formation was perhaps possible in the mouse tissue *in vitro* because of the small size of the tooth germs, which may have allowed sufficient material for amelogenesis to diffuse from the medium into the stratum intermedium. Enamel was nearly always formed at the sides of the molar teeth, where the enamel organ was relatively thin, and never between the cusps where a large amount of stellate reticulum was present; in the incisors, the ectodermal elements on the labial side of the tooth germ where enamel developed, were also thin. Very often there was some slight damage to the enamel organ near where enamel formed, and this was perhaps just enough to bring the medium, with its supply of nutriments, into closer contact with the enamel-forming cells, than in the undamaged tooth germ. This, however, is unlikely to be responsible for all the enamel formed *in vitro*, because of its fairly high rate of occurrence in some batches of cultures, and its complete absence in others.

Although no factors affecting enamel formation came to light in the present investigation, GLASSTONE (1954) has shown that an entirely avian environment can supply the requirements for amelogenesis; she transplanted the molar tooth germs of foetal rats, mice and golden hamsters on to the chorio-allantoic membrane of the chick, and obtained a thick layer of enamel. In this connexion it is interesting to note that MELLANBY and HOLLOWAY (1956) have suggested that mammalian plasma might be better for the cultivation of teeth *in vitro* than cock plasma, on account of the very much lower vitamin A content of the former. They have shown that addition of this vitamin to cock plasma, in which rat tooth germs were being cultivated, had a deleterious effect on the explants and completely suppressed dentine formation.

It is still uncertain whether tooth germs calcify *in vitro* and no proof of it was obtained in the present investigation. GLASSTONE (1938b) found that calcification continued in rabbit incisors in which it had already begun at the time of explantation, but SZABO (1954) has only indirect evidence of its continuance in his cultures. IRVING (1952) said that the dentine of the rat molar does not calcify *in vivo* in the absence of an overlying organic enamel matrix. This might perhaps explain the probable lack of calcification in many of the cultivated molars, but it does not seem to be a very plausible explanation as dentine calcifies *in vivo* on the lingual side of the rodent incisor although no enamel forms there.

The degree of development attained by individual explants was found to vary considerably, probably depending largely on the amount of trauma sustained by the

tooth germ during dissection. In a damaged explant there were sometimes patches of degenerating cells and a few pieces of tissue were so badly damaged that they completely degenerated.

Incisor tooth germs reached the stage of "maximum development" when explanted at the youngest stage (17) from which they could continue to develop *in vitro*. A thick layer of dentine had been laid down after 11 days' cultivation (Fig. 10). Molar tooth germs either developed more slowly, or were not "competent" to differentiate further when explanted at equivalent stages; in a first molar after 10 and in a second molar after 15 days *in vitro* no dentine had been formed. The period of cultivation may not have been long enough; *in vivo* the incisor tooth germs develop more rapidly than the molars.

The "miniature" but histologically normal tooth germs that developed in culture were probably the result of damage sustained by the organ at the time of explantation. GLASSTONE (1952) has shown that halved tooth germs can develop normally from a histological view-point, and that when halved in the early stages of cusp development they regulate to form two complete but small tooth germs.

When LEFKOWITZ, MARDFIN and BODECKER (1954) cultivated foetal rat molar tooth germs in Carrel flasks, they found that the explants only achieved the equivalent of 2–3 days' development measured by *in vivo* standards, and this stage was usually reached in the first 4–6 days of cultivation. Thus the age at which the tissue was explanted was closely related to the stage of development it attained *in vitro*, e.g. tubular predentine was formed in tooth germs explanted on the twentieth and twenty-first days, but not in those excised on the eighteenth and nineteenth days. The development of the tooth germs of the mouse was quite different in that the stages at which they were explanted had almost no effect on the stage of development they reached in culture; molar tooth germs explanted at any stage between the fourteenth day and full-term developed quite a thick layer of dentine and sometimes a little enamel. This seems to be a more advanced stage than that reached by any of the developing teeth cultivated by LEFKOWITZ, MARDFIN and BODECKER (1954).

The youngest tooth germs that it was technically possible to isolate degenerated *in vitro*. That this was mainly due to the inadequacy of the culture conditions, rather than to the tissue not being "determined" for tooth formation, is suggested by the following observations: (i) Tooth germs explanted early on the thirteenth day (Stage 17) developed quite well in hanging drops, but not in watch glasses. The tissue/medium volume relationship is probably important. (ii) Isolated second molar tooth germs do not survive *in vitro* when explanted alone before Stage 33, but they differentiate quite normally from Stage 23, when explanted with the first molar. It is possible that the latter has some specific influence over the second molar which enables it to continue its development, but this seems unlikely as incisor and first molar tooth germs can develop from much earlier stages when part of a larger explant (in which there is no other dental tissue) than when in isolation (HAY, unpublished). (iii) Almost as soon as a tooth germ is able to survive *in vitro*, it is capable of reaching the stage of "maximum development". These findings suggest that in the young tooth germs, it is their size rather than their developmental potency, which is the limiting factor where ability to continue their development *in vitro* is concerned.

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PLATE 1

The sections were stained with Delafield's haematoxylin and chromotrope 2R.

Abbreviations: d.p., dentine papilla; i., lower incisor tooth germ; i.e.e., inner enamel epithelium; l.f.b., lip furrow band; l.r.t., labial root tip of incisor; o.e.e., outer enamel epithelium; t., tongue.

FIG. 1. Coronal section of the lower jaw of a 13 day (Stage 14) mouse foetus, showing the thickenings in the oral epithelium for the incisor tooth germs (i.). ×80.

FIG. 2. Coronal section through part of the tongue (t.) and lower jaw of a 13 day (Stage 17) mouse foetus showing the incisor tooth germs (i.) and the lip furrow band (l.f.b.). ×120.

FIG. 3. Longitudinal section through the lower jaw of a 15 day (Stage 25) mouse foetus showing the relation of the lip furrow band (l.f.b.) to the incisor tooth germ, which is bell-shaped and more developed on its labial than on its lingual side. ×120.

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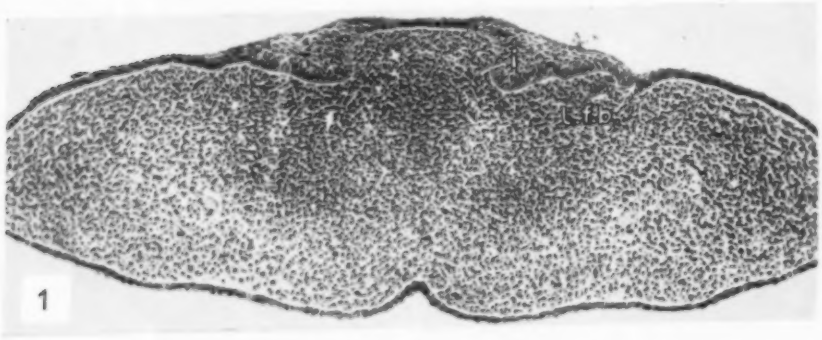

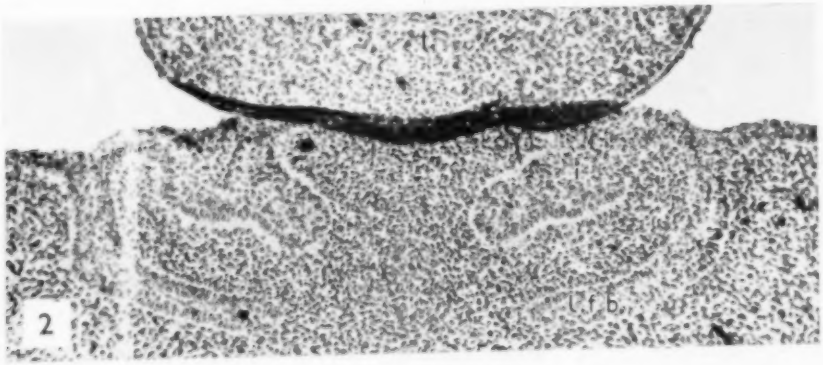

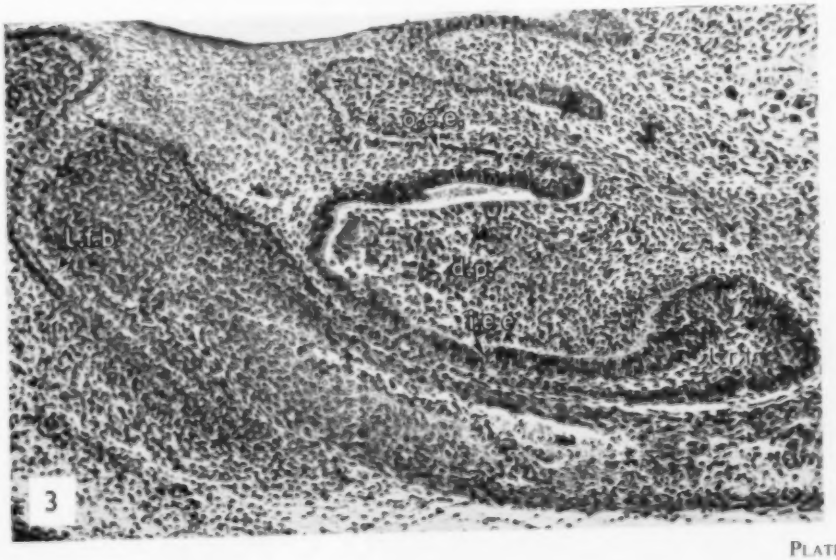

PLATE I

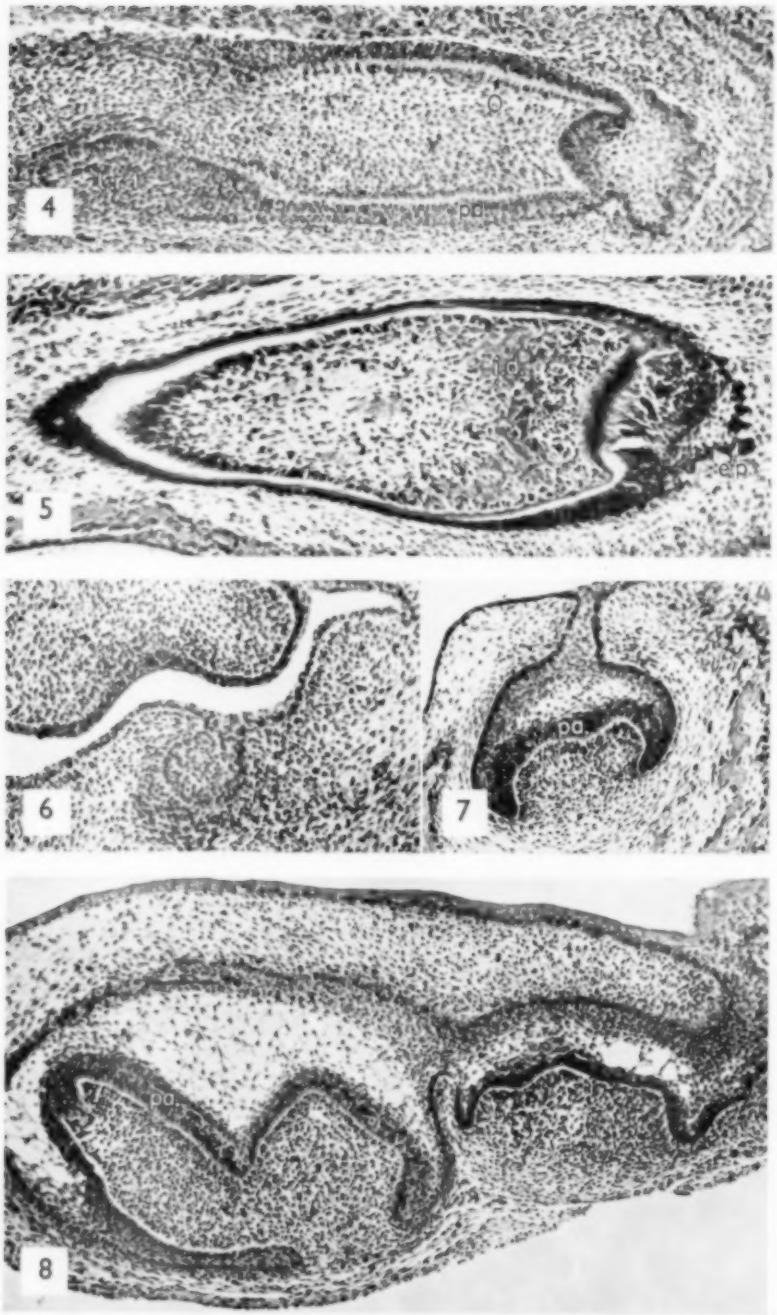

PLATE 2

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PLATE 2

The sections were stained with Delafield's haematoxylin and chromotrope 2R.

Abbreviations: e.p., epithelial papilla; i.d., irregular dentine; l.r.t., labial root tip of incisor; o., odontoblasts; pa., pre-ameloblasts.

FIG. 4. Longitudinal section through the lower incisor tooth germ of a 16 day (Stage 28) mouse foetus. Pre-ameloblasts (pa.) and odontoblasts (o.) are present. ×105.

FIG. 5. Tangential section through the lower incisor of an 18 day (Stage 35) mouse foetus showing epithelial papillae (e.p.) and irregular dentine (i.d.). ×105.

FIG. 6. Coronal section through the molar region of one side of the lower and part of the upper jaw of a 13 day (Stage 19) mouse foetus. Mesenchyme can be seen condensing round the bud-shaped first molar tooth germ. ×105.

FIG. 7. Transverse (coronal) section of the first lower molar of a 15 day (Stage 24) mouse foetus, showing an early stage in cusp formation. ×105.

FIG. 8. Longitudinal section through the first and second lower molar tooth germs of a 17 day (Stage 32) mouse foetus. Many mitotic figures can be seen in the pre-ameloblasts (pa.) of the first molar. ×98.

PLATE 3

The sections were stained with Delafield's haematoxylin and chromotrope 2R.

Abbreviations: a., ameloblasts; d., dentine; o, odontoblasts; o.e.e., outer enamel epithelium.

FIG. 9. Transverse (coronal) section of the first lower molar of a 1 day old mouse, showing the blood spaces in the stellate reticulum. ×70.

FIG. 10. The labial amelo-dentinal region of a culture of an incisor tooth germ explanted at the stage shown in Fig. 2 (thirteenth day; Stage 17) when the enamel organ had just begun to invaginate, and cultivated for 11 days *in vitro*. Odontoblasts (o.), ameloblasts (a.) and dentine (d.), all of normal appearance, developed. ×525.

FIG. 11. Longitudinal section through a culture of an incisor tooth germ explanted at the stage shown in Fig. 4 and cultivated for 11 days *in vitro*. A layer of dentine (d.) has been formed; it is rather abnormal at the incisal end, probably due to damage to the enamel organ. ×105.

FIG. 12. Models (×70) showing enlargement of the dentine papilla *in vitro* and *in vivo*. A. Model of the dentine papilla of the first lower molar of a 16 day (Stage 28) mouse foetus. A longitudinal section of this tooth germ is shown in Fig. 15. B. Model of a similar dentine papilla to A after 11 days *in vitro*. Transverse section shown in Fig. 13. C. Model of the dentine papilla of the first lower molar of a 7 day old mouse, i.e. 11 days older than A. Three groups of cusps can be seen in it.

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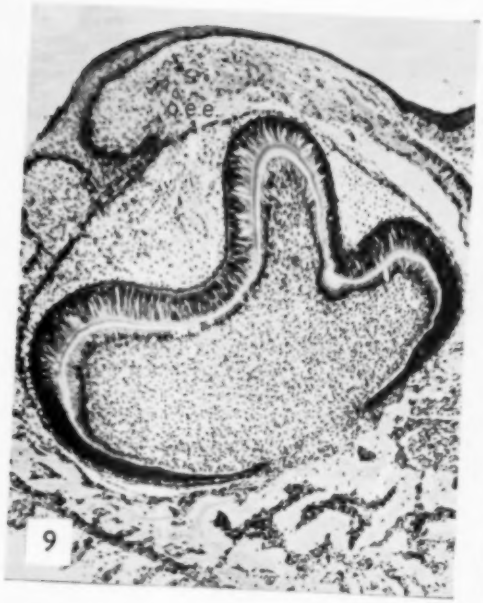

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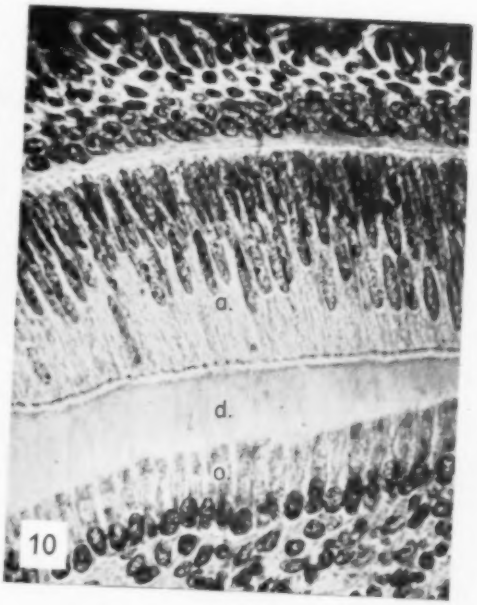

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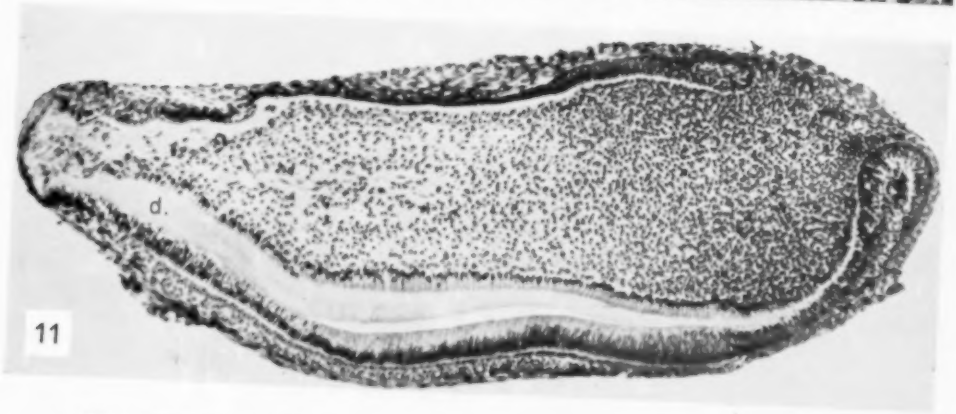

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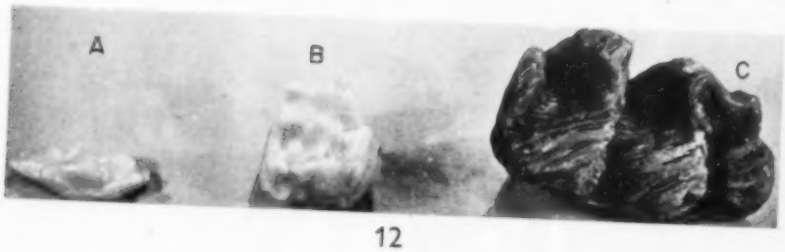

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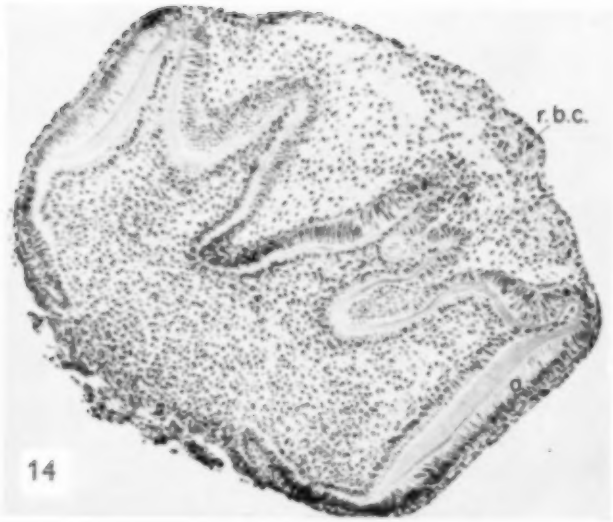

PLATE 4

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PLATE 4

The sections were stained with Delafield's haematoxylin and chromotrope 2R.

Abbreviations: a., ameloblasts; d., dentine; k., keratinized epithelium; r.b.c., red blood corpuscles.

FIG. 13. Transverse section of a culture of the first lower molar explanted at the stage shown in Fig. 15 (sixteenth day; Stage 28) and cultivated for 11 days *in vitro*. Ameloblasts and odontoblasts have differentiated and a layer of dentine (d.) has been laid down. A small piece of the oral epithelium has keratinized (k.) close to the tooth. ×120.

FIG. 14. Longitudinal section of a culture of a first lower molar explanted at the stage shown in Fig. 16 (seventeenth day; Stage 31) and cultivated for 10 days *in vitro*. Odontoblasts and ameloblasts (a.) have developed, and the absence of the latter over the tips of the cusps can be seen. Dentine has been laid down. The remains of some red blood corpuscles (r.b.c.) are present at the edge of the explant. ×120.

PLATE 5

The sections were stained with Delafield's haematoxylin and chromotrope 2R.

Abbreviations: d., dentine; e., enamel; s.i., stratum intermedium.

FIG. 15. Longitudinal section of the first lower molar of a 16 day (Stage 28) mouse foetus. ×120.

FIG. 16. Longitudinal section of the first lower molar of a 17 day (Stage 31) mouse foetus. The stellate reticulum and stratum intermedium (s.i.) are well developed and three groups of cusps can be seen. ×120.

FIG. 17. Part of a longitudinal section of an incisor tooth germ explanted at Stage 28 and cultivated for 16 days. Enamel (e.) has developed; the tubular structure of the dentine (d.) can be seen; the enamel organ appears to be reduced to three or four layers of squamous cells. ×540.

FIG. 18. Part of a longitudinal section of an incisor tooth germ explanted at Stage 28 and cultivated for 9 days *in vitro*. The ameloblasts which appear to have laid down enamel (e.) are not much shorter than their non-functional neighbours. ×540.

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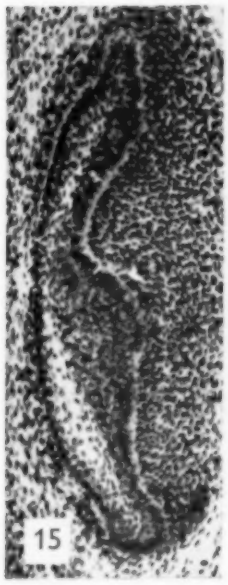

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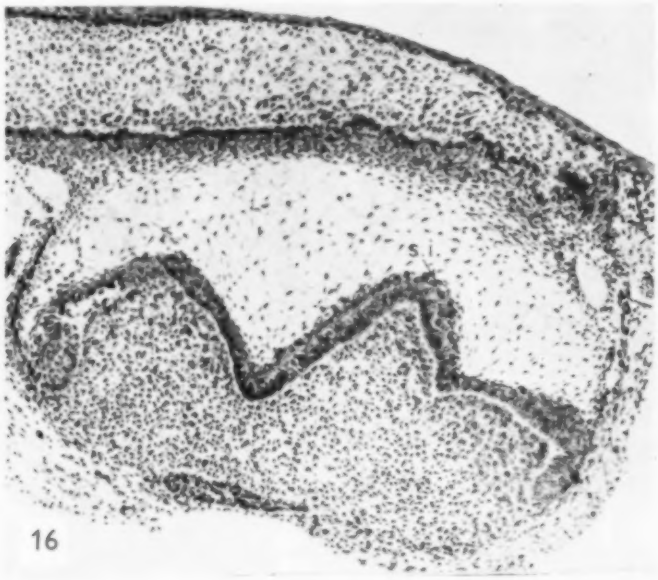

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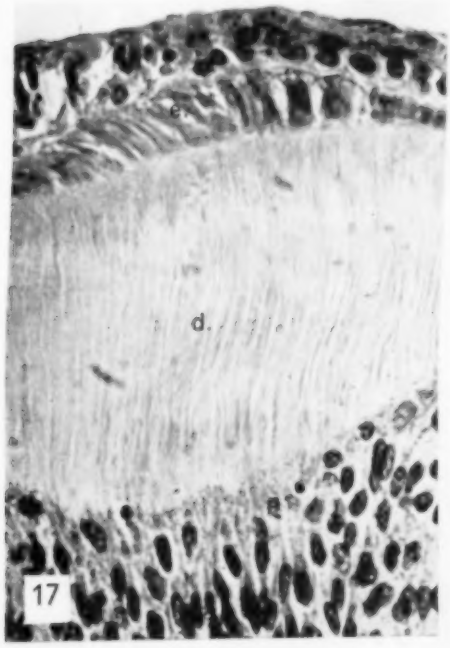

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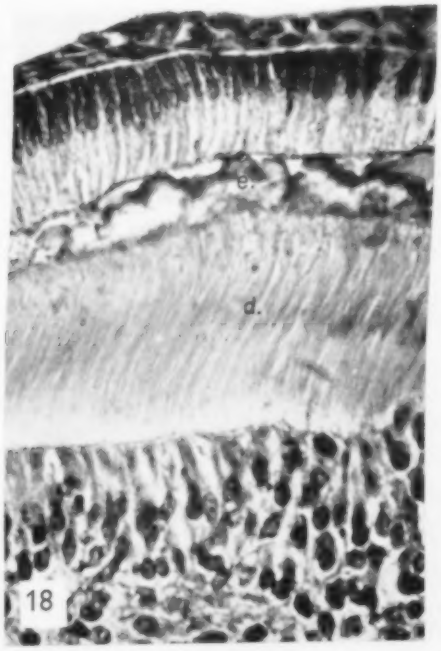

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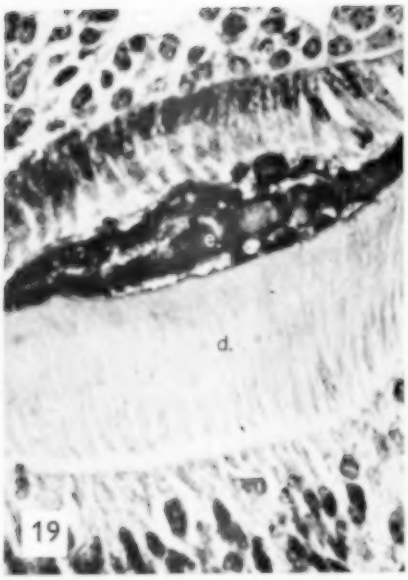
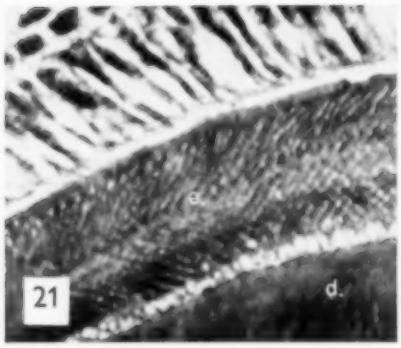
PLATE 5





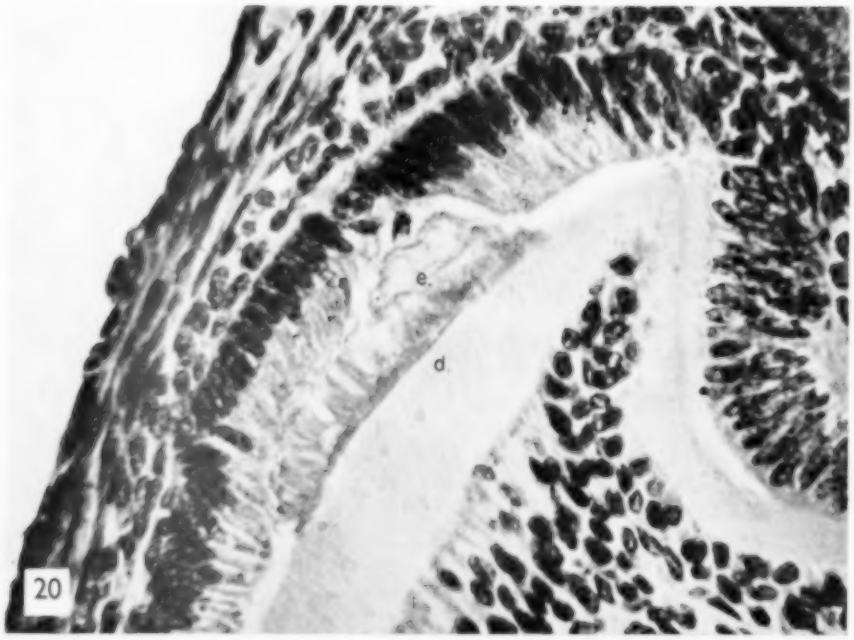


PLATE 6

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PLATE 6

The sections were stained with Delafield's haematoxylin and chromotrope 2R.

Abbreviations: d., dentine; e., enamel.

FIG. 19. Part of a longitudinal section of an incisor tooth germ explanted at Stage 24 and cultivated for 14 days *in vitro*. A globular type of enamel (e.) has been laid down. ×608.

FIG. 20. Part of a longitudinal section of a first molar tooth germ explanted at Stage 30 and cultivated for 11 days *in vitro*. Some enamel (e.) can be seen on the outer edge of a cusp near its tip. The length of the ameloblasts appears to be inversely proportional to the thickness of the underlying enamel. ×608.

FIG. 21. Enamel (e.) as it appears in a transverse section of a partially decalcified incisor of a 4 day old mouse. (For comparison with Figs. 17–20). ×608.

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ZEITLICHER VERLAUF UND ERFASSUNG DER KARIES IN EINER TIEREXPERIMENTELLEN STUDIE

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Zusammenfassung—Es wurde der zeitliche Verlauf der Karies über eine Versuchsperiode von 0–180 Tagen bei kariesempfindlichen entwöhnten Wistar-Ratten verfolgt. Die gleichzeitige Anwendung einer histologischen Auswertungsmethode an unentkalkten Molarenschliffen und einer makroskopischen Auswertungsmethode zur Feststellung des Karies-Ausmasses gab Aufschluss über Indikation und Abgrenzung beider Methoden. Initialstadium und beginnende Progression der Karies konnten mit einer mikroskopischen Auswertungsmethode innerhalb einer Versuchsdauer von 20 Tagen einwandfrei registriert werden. Bei Anwendung dieser Methode konnte nach 20 Versuchstagen kein weiterer Kariesanstieg erfasst werden, obwohl auf Grund einer makroskopischen Auswertungsmethode das Karies-Ausmass zunahm. Für die Registrierung einer weiteren Ausbreitung der Karies unter Bildung von Kavitäten war daher die Anwendung einer makroskopischen Auswertungsmethode erforderlich.

Abstract—The development of dental caries was observed continuously in groups of caries-susceptible Wistar rats. The experimental periods lasted from weaning until 1, 2, 3, 6, 9, 12, 15, 20, 40, 60, 120 and 180 days, respectively, during which time the animals received a cariogenic diet. Two methods of caries evaluation were used simultaneously in order to observe initiation and progression of the caries process. A method using undecalcified serial sections of the molar teeth was found to be suitable for the evaluation of initial enamel caries and of initial dentine caries within an experimental time of 20 days. It was shown that the histologic method used should be limited to the evaluation of initial caries. Further progression of caries and cavitation was determined by a method using a binocular microscope of 10× magnification. Under these experimental conditions the evaluation of progressive carious lesions and cavities necessitates an experimental period of 80–120 days.

EINLEITUNG

ZUR Feststellung einer karieshemmenden oder kariesfördernden Wirkung bestimmter Kostformen oder Kostbestandteile wenden verschiedene Arbeitskreise im Tierexperiment kurzfristige Kariesstudien an (LOSEE, GERENDE und NEMES, 1955; MARTHALER, MÜHLEMANN und KÖNIG, 1957; STEINMAN, HEWES und WOODS, 1959; DALDERUP, 1959; MELZER und KINKEL, 1960). Die histologische Kariesauswertung an unentkalkten Serienschliffen der Rattenmolaren erfolgt hierbei je nach Planung und Methodik nach einer Experimentalzeit von zwischen 10 Tagen und 6 Wochen. Bei der Auswertung werden das Initialstadium und die beginnende Progression der Karies erfasst.

Mit makroskopischen Auswertungsmethoden werden unter Anwendung der binokularen Prismenlupe bestehende Kavitäten registriert. Hierfür sind im allgemeinen jedoch Experimentalzeiten von wenigstens 3–4 Monaten erforderlich.

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Nach Beobachtungen von DALDERUP (1959, 1961) kann bei der histologischen Kariesauswertung an unentkalkten Schliffen eine Farbaufnahme im Schmelz der Fissuren als Initialkaries registriert werden, ohne dass es späterhin zur Progression der Karies und zur Bildung einer Dentinkaries und Kavitation kommt. Die Autorin führt dieses Phänomen auf eine stärkere Affinität des noch nicht ausgereiften Schmelzes zu der verwandten Fuchsin-Lösung zurück. Diese Festellung muss zu dem Schluss führen, dass eine Auswertungsmethode, welche nur das Initialstadium der Karies erfasst, unsicher ist, da eine Unterscheidung zwischen beginnender Schmelzkaries und nicht ausgereiftem Schmelz auf Grund der Färbbarkeit nicht möglich ist.

Die vorliegende Studie versucht zur Klärung dieses Problems beizutragen. Es wird der zeitliche Verlauf der Karies vom Initiationsstadium über die Progression bis zur Registrierung der Kavitäten verfolgt, unter gleichzeitiger Anwendung einer mikroskopischen und einer makroskopischen Kariesauswertung.

MATERIAL UND METHODE

Gruppen von je 6–7 entwöhnten Ratten eines kariesempfänglichen Wistar-Stammes erhielten im Alter von 21 Tagen die von STEPHAN und HARRIS (1955) beschriebene kariogene Kost Nr. 580, bestehend aus 66% Rohrzucker, 32% Magermilchpulver und 2% Trockenleber. Kost und Trinkwasser wurden ad libitum verabreicht. Die Tierhaltung entsprach den früher mitgeteilten Angaben (MELZER und KINKEL, 1960). Die Versuchsperioden, beginnend im Alter der Tiere von 21 Tagen, betrugen 1, 2, 3, 6, 9, 12, 15, 20, 40, 60, 120, 180 Tage. Eine weitere Gruppe von 6 Ratten wurde bereits am Tage der Entwöhnung im Alter von 21 Tagen getötet, die anderen Tiere jeweils am Ende der entsprechenden Experimentalzeit.

Mikroskopische Methode

Die mikroskopische Kariesauswertung an unentkalkten Schliffen erfolgte nach der von MARTHALER, MÜHLEMANN und KÖNIG (1957) angegebenen Methode.

Mit dem Schneideapparat nach JANSEN (1950), von DALDERUP und JANSEN (1955) und von BÜTTNER (1956) modifiziert und beschrieben, wurden 5–7 sagittale Serienschnitte (etwa 120 μ dick) von den unteren Quadranten des Rattenkiefers hergestellt. Die Serienschnitte ermöglichen, die Fissuren in ihrer buccolingualen Ausdehnung zu überblicken sowie auch kurze Fissuren zu erfassen. Erkennen und Abgrenzen vorhandener kariöser Prozesse wird durch Anfärben (etwa 10 sec) mit dem Schiff'schen Reagens ermöglicht bzw. erleichtert. Die beobachteten Läsionen wurden nach Schweregraden in eine Auswertungskarte eingetragen. Es wurde mit einem binokularen Mikroskop bei 60-facher Vergrösserung gearbeitet.

Diese Methode unterscheidet 3 Schweregrade der Läsionen:

Grad A: Farbreaktion auf den Schmelz beschränkt.

Grad B: Farbreaktion an der Schmelz-Dentin-Grenze und halbmondförmige Ausbreitung unter dem Schmelz (frühestes definitives Kariesstadium).

Grad C: Abhebung des Schmelzes, beginnende Kavitation.

Für die Ermittlung des Karies-Ausmasses pro Versuchsgruppe bedienen sich MARTHALER *et al.* (1957) folgender Berechnung: Es wird die während der gesamten Versuchsperiode aufgetretene

Anzahl Läsionen der verschiedenen Grade ermittelt ("total durchlaufen"), d.h. Grad A ist gleich der totalen Läsionszahl (A+B+C), denn die schwereren Grade B and C hatten auch Grad A zu durchlaufen. Entsprechend setzt sich Grad B aus B+C zusammen, Grad C bleibt Grad C.

Makroskopische Methode

Die parallel laufende makroskopische Kariesauswertung stützte sich auf die Methode von MUHLER, NEBERGALL und DAY (1954).

Bei dieser Methode sind im Karies-Ausmass neben der Zahl der Läsionen auch 3 verschiedene Schweregrade enthalten (MUHLER und DAY, 1950; MUHLER, NEBERGALL und DAY, 1953). Die Methode wurde von ROZEIK, CREMER und HANNOVER (1956) in der Weise modifiziert, dass noch ein Schweregrad 4 für zusammengeflossene Kavitäten hinzugefügt wurde. Da die Diagnose von Kavitäten des Schweregrades 1 unsicher ist, wurde in der vorliegenden Arbeit bei der Aufstellung des Kariesausmasses auf diesen Grad verzichtet. Entsprechend der Überlegung bei der mikroskopischen Auswertung wurde auch bei der makroskopischen Auswertung die während der gesamten Versuchsperiode aufgetretene Anzahl von Kavitäten der verschiedenen Grade ermittelt, somit setzt sich das Karies-Ausmass aus 2+3+4 zusammen.

Die einzelnen Grade sind wie folgt klassifiziert:

Grad 1: beginnende Fissurenkaries, schwer erkennbar und unsicher.
Grad 2: deutliche Fissurenkaries, Defekt im Schmelz.
Grad 3: Kavitation, Ausbreitung der Läsion im Dentin.
Grad 4: grosse Kavitäten, bei denen bereits 2 oder 3 Fissuren verloren gegangen sind und Teile eines Zahnes oder der gesamte Zahn zerstört sind.

Die makroskopische Auswertung erfolgte mit einer binokularen Prismenlupe bei 10—facher Vergrösserung.

ERGEBNIS

Die Daten der mikroskopischen Kariesauswertung nach den Versuchstagen 0–20 sind aus Tabelle 1 und aus den graphischen Darstellungen Fig. 1 und 2 zu ersehen. Bereits am Versuchstag 0 (Kariesauswertung im Alter der Tiere von 21 Tagen am Tage der Entwöhnung) wurden Läsionsgrade A festgestellt. Wie in Fig. 1 dargestellt,

TABELLE 1. KARIES-AUSMASS NACH MIKROSKOPISCHER AUSWERTUNG DER LÄSIONSGRADE A, B UND C INNERHALB VON 20 VERSUCHSTAGEN

Tierzahl	Versuchstage	Läsionsgrade		
		A	B	C
6	0	14,5±0,8*	0,5±0,3	—
6	1	14,2±0,5	1,4±0,5	—
6	2	16,4±0,3	2,2±0,6	—
6	3	17,7±0,4	7,3±0,7	—
6	6	17,1±0,4	11,8±0,9	0,5±0,3
6	9	20,5±1,0	13,2±0,3	1,2±0,5
6	12	20,2±0,8	11,2±0,9	1,8±0,6
6	15	21,5±0,4	12,5±0,4	3,2±0,5
6	20	21,4±0,8	13,0±1,7	4,8±0,6

* Fehler des Mittelwertes.

erfolgte ein stetiger leichter Anstieg der A-Grade bis zum 20. Versuchstag. Die ersten Läsionsgrade B (beginnende Dentinkaries) konnten ebenfalls bereits am Versuchstag 0 and 1 registriert werden, wenn auch in viel geringerer Anzahl als

A-Grade. Läsionen vom Schweregrad C (Abhebung des Schmelzes, beginnende Kavitation) wurden ab 6. Versuchstag beobachtet. Vom 2. Versuchstag an erfolgte ein schneller Anstieg der B-Grade. Dies dürfte ein Zeichen dafür sein, dass die Mehrzahl der anfänglich gefundenen A-Grade in Läsionsgrade B übergingen.

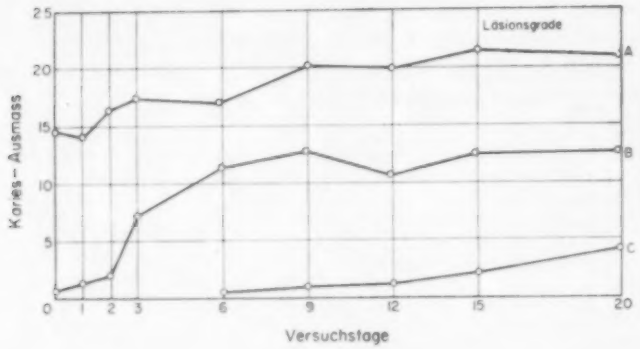

FIG. 1. Karies-Ausmass nach mikroskopischer Auswertung der Läsionsgrade A, B and C innerhalb von 20 Versuchstagen.

In Fig. 2 ist der Verlauf der Läsionsgrade vom Versuchstag 0–20 für die einzelnen Molaren dargestellt. Die ersten B-Grade wurden zunächst an den ersten Molaren, die im Alter der Tiere von etwa 19 Tagen durchbrechen, festgestellt.

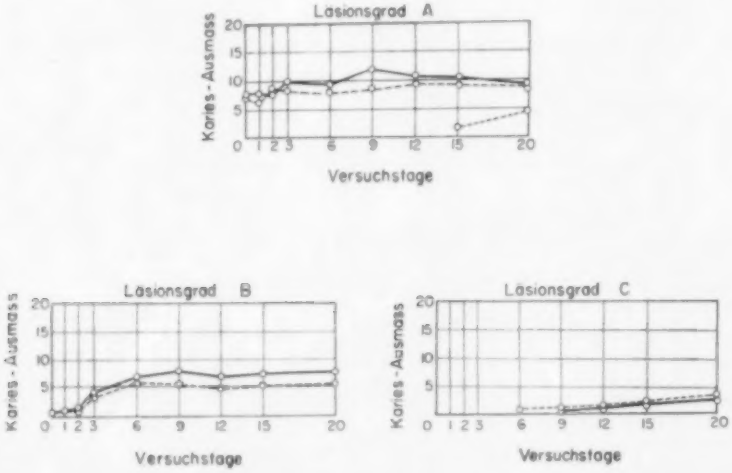

FIG. 2. Mikroskopische Kariesauswertung bei den einzelnen Molaren während 20 Versuchstagen. 1. Molar: durchgezogene Linie. 2. Molar: Strich-Linie. 3. Molar: Punkt-Linie.

An den 2. Molaren wurden die ersten B-Grade nach dem 1. Versuchstag und an dem 3. Molaren (Durchbruch bei einem Alter von 31–34 Tagen) nach 20 Versuchstagen, d.h. etwa am 40. Lebenstag, notiert. Die ersten C-Grade traten am 6. Versuchstag am 2. Molaren auf, nach 9 Versuchstagen am 1. Molaren.

Tabelle 2 zeigt das Ergebnis der weiteren *mikroskopischen* Auswertung nach 20–180 Versuchstagen. Vom 20. Versuchstag an war hier für die einzelnen Läisonsgrade kein signifikanter Kariesanstieg mehr zu verziechnen, da mit dem Erreichen des Grades C lediglich die Kavitation, nicht aber ein Fortschreiten des Kariesprozesses und die Bildung grösserer Kavitäten registriert wurde.

TABELLE 2. KARIES-AUSMASS NACH MIKROSKOPISCHER AUSWERTUNG DER LÄSIONSGRADE A, B UND C INNERHALB VON 20 BIS 180 VERSUCHSTAGEN

Tierzahl	Versuchstage	Läsionsgrade		
		A	B	C
7	20	17,5*	13,2	11,2
7	40	19,2	11,8	8,8
7	60	18,8	11,3	8,2
6	120	22,2	13,8	11,8
7	180	21,6	14,6	10,7

* Aus Übersichtsgründen wurde auf die Angabe der Mittelwertsfehler in dieser Tabelle verzichtet. Die Fehler der Mittelwerte bewegen sich in der gleichen Grössenordnung wie in Tabelle 1.

Bei Anwendung der *makroskopischen* Auswertungsmethode wurden bis zum 40. Versuchstag keine Kavitäten der Schweregrade 2, 3 und 4 beobachtet Am 40. Versuchstag treten die ersten auswertbaren Kavitäten auf. Nunmehr konnte auf Grund dieser Auswertungsmethodik, welche auch das Ausmass bestehender Kavitäten berücksichtigt, ein steter Anstieg im Kariesbefall bis zum Ende der Experimentalzeit von 180 Tagen registriert werden.

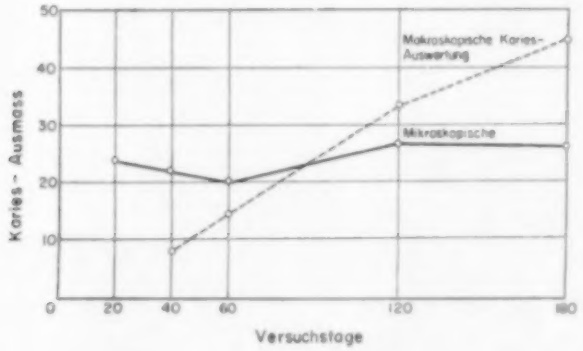

FIG. 3. Vergleich der makroskopischen mit der mikroskopischen Karies-auswertung. Karies-Ausmass während Versuchsperioden von 20–180 Tagen.

In Tabelle 3 und Fig. 3 werden die bei mikroskopischer und makroskopischer Kariesauswertung erzielten Ergebnisse nebeneinander dargestellt. Hierfür benutzten wir für beide Auswertungsmethoden nur die Stadien definitiver Karies, also die Grade B+C für die mikroskopische und die Grade 2+3+4 für die makroskopische Auswertung. Aus dem Verlauf der Kurven in Fig. 3 ist zu ersehen, dass bei Anwendung der mikroskopischen Auswertungsmethode nach 20 Tagen kein weiterer Anstieg im Kariesbefall festgestellt werden konnte. Makroskopisch auswertbare Karies zeigte sich frühestens nach einer Versuchsdauer von 40 Tagen. Die weitere Progression der Karies offenbart sich in einem steten Anstieg der Kurve bis zu 180 Tagen Versuchsdauer.

TABELLE 3. GLEICHZEITIGE MAKROSKOPISCHE UND MIKROSKOPISCHE KARIESAUSWERTUNG INNERHALB VON 20–180 VERSUCHSTAGEN

Versuchstage	Läsionsgrade B+C (mikroskopisch)	Läsionsgrade 2+3+4 (makroskopisch)
20	24,4±1,6*	—
40	20,6±3,2	8,5±2,3
60	19,5±3,3	14,8±4,2
120	25,6±4,3	34,3±7,7
180	25,3±5,2	46,0±7,3

* Fehler des Mittelwertes.

DISKUSSION

Kurzfristige Kariesstudien bieten den Vorteil, dass innerhalb kleiner Zeiträume die Wirkung verschiedener für eine Karieshemmung oder Kariesausbreitung verantwortliche Faktoren beobachtet werden kann. Die histologische Auswertungsmethode an unentkalkten Serienschliffen, wie sie von MARTHALER *et al.* (1957) beschrieben wurde, erfasst jedoch nur das Initiationsstadium der Karies und eine beginnende Kariesprogression, während die weitere Progression unter Ausbildung grösserer Kavitäten nicht mehr registrierbar ist. Somit muss die Anwendung der Methode auf Kariesstudien beschränkt bleiben, in denen eine Initiationshemmung untersucht werden soll. MARTHALER *et al.* (1957) wiesen, wie auch DALDERUP (1959), darauf hin, dass eine Farbreaktion des Schmelzes mit der für die Auswertung benutzten Farblösung auch von unreifem Schmelz gegeben wird, also nicht unbedingt das Initialstadium der Karies darstellen muss. Deshalb sollten für die zahlenmässige Erfassung des Karies-Ausmasses nur Läsionen zu Grunde gelegt werden, bei denen bereits eine Mitbeteiligung des Dentins sichtbar ist. Die Beobachtungen in dieser Studie lassen allerdings den Schluss zu, dass sich bei den vorliegenden Versuchsbedingungen an Stellen einer Schmelzfärbung späterhin auch Kavitäten bilden.

Die mikroskopische Auswertung an unentkalkten Molarenschliffen erwies sich bei dem von uns verwandten kariesempfindlichen Rattenstamm als eine brauchbare und zuverlässige Methode zur Feststellung der Initiation und der beginnenden Progression der Karies bei einer Versuchsdauer von 20 Tagen.

Nach weiterer Ausbreitung der Karies und nach der Bildung von Kavitäten ist die Anwendung einer makroskopischen Auswertungsmethode angezeigt. Bei unseren Versuchsbedingungen kann hierbei die Auswertung zu einem Zeitpunkt zwischen 80 und 120 Tagen erfolgen.

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RECURRENT "FEVER BLISTER" AND "CANKER SORE"

TESTS FOR HERPES SIMPLEX AND OTHER VIRUSES WITH MAMMALIAN CELL CULTURES

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Abstract—Specimens from lesions of eleven patients with recurrent herpes labialis and thirty-one with recurrent aphthous ulcerations were tested for herpes simplex and other viruses in cultures of rabbit kidney, monkey kidney, HeLa, and human skin cells. Of twenty-five herpetic lesions cultured, thirteen, representing seven patients, yielded herpes simplex virus; none of the twenty-five gave evidence of any other virus. None of 100 recurrent aphthous ulcertations showed viral activity by cytopathic effects or haemadsorption tests in primary cultures and subcultures.

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INTRODUCTION

RECURRENT herpes labialis, commonly referred to as "fever blister" or "cold sore", has been reported to occur in 38 per cent of the population (SHIP *et al.*, 1960). Probably more than 50 per cent are susceptible, since on the average three-fourths of adults show continued activity of the causative virus, herpes simplex or *Herpesvirus hominis*, by maintaining significant levels of homologous circulating antibody (BURNET and LUSH, 1939). This syndrome is characterized by recurrent episodes of vesicle formation on the lips, usually on or near the mucocutaneous junction. The vesicle appears after a prodromal period of 24–48 hr, marked by a peculiar hyperaesthesia and burning sensation in the region of the developing lesion. Localized oedema and erythema of the lip are the initial clinical findings. During the following 24 hr a round-to-oval vesicle appears, containing a clear, colourless fluid. Finally the vesicular membrane disrupts, leaving an ulceration with an irregular margin. The base of the ulcer is serohaemorrhagic and not surrounded by an erythematous aureole. The base coagulates and healing proceeds uneventfully from both margin and base. Within 7–10 days after the onset of symptoms, the lesion heals completely, leaving no scar. The frequency of recurrences varies widely, from one lesion every few years to weekly or even daily episodes of labial ulceration.

Herpes simplex virus has been demonstrated in as many as 90 per cent of cases by inoculation of vesicular fluid or swabs of lesions upon the chorio-allantoic membranes of embryonated eggs or upon the scarified corneas of rabbits (BLANK *et al.*, 1950). Serological studies show that in the majority of cases serum antibody titres to herpes simplex virus are significantly high initially and do not change during convalescence (DASCOMB, ADAIR and ROGERS, 1955). The finding of multinucleate giant epithelial cells exhibiting characteristic intranuclear changes in biopsy specimens of early lesions is diagnostic and provides additional evidence

of infection with herpes simplex virus (Blank *et al.*, 1951), though it does not differentiate this infection from herpes zoster and varicella. Apparently, this virus first enters the body early in life, commonly causing primary acute herpetic gingivostomatitis, and subsequently persists in a latent state. A remarkable feature of this common infection is the variety of nonspecific stimuli that apparently activate recurrences of cold sores, e.g. fever, natural or induced emotional stress, nerve injury, menstruation, allergy, sunlight, and reduced general resistance. (For a general account of herpetic infections, see Scott, 1959).

Recurrent aphthous stomatitis, commonly referred to as "canker sore", is characterized by recurrences of painful ulcerations on the oral mucous membranes. This condition has been designated also as "vesicular stomatitis", "maculofibrinous stomatitis", "dyspeptic ulcers", "periadenitis mucosa necrotica recurrens", "neurotic ulcers", and many other terms. One study indicates that 19 per cent of the population is affected, females more frequently than males (Sircus, Church and Kelleher, 1957). In another survey, 55 per cent of a professional-school population reported experience with this condition (Ship *et al.*, 1960).

During a prodromal period lasting from 1 to 24 hr, a sensation of hyperaesthesia and burning is felt in the affected mucosa, similar to that experienced prior to the appearance of a cold sore on the lips. Initially, a small erythematous macule appears, which develops a central blanched region that breaks down within several hours to form a shallow crateriform ulcer about 1 cm in diameter with a regular, sharply delineated margin and a greyish-yellow fibrinous base, surrounded by a narrow, well-marked erythematous aureole. Aphthous ulcers are extremely painful; consequently, eating, speaking and other manoeuvres necessitating facial movements can become difficult. The ulcer persists for 7–12 days and heals slowly from the margin toward the centre, leaving no scar. Recurrences may be continual, with as many as several dozen ulcers at any given time, or irregular, with only one lesion every few years.

Primary aetiological significance in this disease has been attributed to a multitude of factors, such as emotional trauma, vitamin deficiency, food or drug allergy, endocrine disturbances, and local physical trauma (Sircus, Church and Kelleher, 1957). The recurrent nature of this syndrome, the grossly similar appearance of its lesions to cold sores, its apparent provocation by a variety of non-specific stimuli, its complete unresponsiveness to all sorts of systemic and local therapy, and the lack of a more suitable explanation have contributed to the view that recurrent canker sores are manifestations of infection with herpes simplex virus. However, attempts to isolate this, or any other virus from aphthous ulcers have been almost uniformly unsuccessful in several laboratories (Driscoll *et al.*, 1959). Furthermore, no one has reported the finding of cellular changes typical of herpetic infection in biopsy specimens from recurrent canker sores (Driscoll *et al.*, 1959). Serological evidence likewise does not confirm an aetiological role of herpes virus. Stark, Kibrick and Weisberger (1954) found antibody to herpes simplex virus by complement fixation in sera of 80 per cent of thirty control subjects, but in only 55 per cent of twenty-nine patients with recurrent aphthae. The median titre of the controls was from two to four times that of the patients. In each group, all those with a

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positive or questionable history of herpes had antibody, compared to a third of those with no history of herpes. The striking difference seems to be that twenty of the twenty-nine patients gave a negative herpetic history, compared to nine of the thirty controls. BLANK *et al.* (1950) demonstrated herpetic antibodies by neutralization tests in sera of all of nineteen patients with fever blisters and thirty-eight patients recovering from primary acute herpetic gingivostomatitis, but in only eight of thirteen patients with canker sores. DODD and RUCHMAN (1950) found significant levels of herpes-neutralizing antibody in only two of eleven patients convalescing from canker sores, as compared with high titres in all of eight patients convalescing from primary herpetic infection.

The uncertain aetiology of recurrent canker sores made it appropriate to attempt the isolation of viruses from these lesions in cultures of several types of mammalian cells known to be susceptible to a variety of such agents. Specimens from fever blisters were included to test the efficacy of the procedures used.

MATERIALS AND METHODS

Patient material

Patients with recurrent canker sores and with recurrent fever blisters were seen in the Dental Clinic, Clinical Center, National Institutes of Health. Multiple samples were taken from canker sores in various stages of formation. The lesion was initially dried with a stream of warm air and the base of the ulcer was débrided with sterile dental hand instruments to give the desired specimen. All fever blisters were cultured during the first or second day of formation. The site was cleansed with alcoholic Zephiran (brand of benzalkonium chloride U.S.P.) and fluid was aspirated into a sterile syringe through a hypodermic needle inserted through the intact vesicular membrane. All specimens were immediately transferred to tubes of culture medium labelled only with code numbers and taken to the laboratory for culturing. In a few cases, duplicate samples were cultured by another virus laboratory, which reported identical results.

Cultural procedures

Solutions and media. Phosphate buffered saline (PBS) was prepared as described by DULBECCO and VOGT (1954). Trypsin (1 : 300, Nutritional Biochemicals Corp.) was made up in 0·25% solution in saline A (MARCUS, CIECIURA and PUCK, 1956) and sterilized by Seitz filtration. Lactalbumin hydrolysate medium consisted of a 0·5% solution of lactalbumin hydrolysate (Nutritional Biochemicals Corp.) in Earle's balanced salt solution (EARLE, 1943), sterilized by Seitz filtration under pressure and stored at 4°C. Eagle's basal medium (EAGLE, 1955) was obtained as a concentrate from the Media Section, National Institutes of Health, and diluted with sterile triple-distilled water. Serum as required variously and antibiotics (penicillin G, 100 units/ml; streptomycin, 100 μg/ml; tetracycline, 100 μg/ml; nystatin, 100 units/ml) were added to these media just prior to use.

Rabbit kidney cell culture. Suspensions of rabbit kidney cells were prepared by YOUNGNER's technique (1954), extensively modified. Albino rabbits from 8 to 10

weeks of age were exsanguinated and their kidneys were removed aseptically. The cortex was dissected out, sliced, and washed three times in a 90 mm petri dish with PBS containing the antibiotics listed previously, to remove as much blood as possible. The washed fragments were transferred to a 500-ml trypsinizing flask (Model 14-2251, Bellco Glass Co., Vineland, N.J.) containing a Teflon-coated magnetic stirring bar and agitated gently for 10 min in 100–150 ml of trypsin solution, pre-warmed to 37°C. The supernatant, containing mostly red blood cells and debris, was discarded. The tissue was then extracted similarly seven to nine times with fresh portions of trypsin solution. The extracts were filtered into 200-ml centrifuge bottles through gauze to remove debris. The cells were sedimented by low speed centrifugation and resuspended in 40–55 ml of lactalbumin hydrolysate medium supplemented with 2 per cent calf serum, which terminated tryptic activity. The cells were collected finally in 40-ml graduated conical centrifuge tubes. The average yield was 2·8 ml of packed cells per kidney. These cells were suspended in 150 vol of lactalbumin hydrolysate medium supplemented with 8 per cent calf serum, yielding an average viable count of $1{\cdot}5\times10^5$ cells/ml. Portions (0·9 ml) of these suspensions were dispensed in rubber-stoppered 16×150 mm test tubes, which were then incubated in a stationary, nearly horizontal position for 4 days at 37°C. The supernatants were then withdrawn and replaced with 1·5 ml portions of Eagle's medium supplemented with 10 per cent inactivated rabbit serum. Viral inocula were introduced on the fifth or sixth day.

Monkey kidney cell cultures. Cultures of monkey kidney cells were prepared like those from rabbit kidneys, except that the packed cells were suspended in 200 vol of lactalbumin hydrolysate medium supplemented with 3 per cent calf serum. This medium was used throughout the experiment. Also, only 0·5 ml portions of cell suspension were dispensed per tube.

HeLa cell cultures. A substrain of the S3 HeLa clone of PUCK, MARCUS and CIECIURA (1956) was maintained as a stock culture in Eagle's medium supplemented with 10 per cent inactivated horse serum. Tube cultures were started with 0·5 ml portions of stock suspensions containing an average of 10^5 cells. On the following day, the supernatant was removed and replaced with 1·5 ml of fresh medium and the viral inoculum was introduced.

Human skin cell cultures. The strain of human skin cells of PERRY *et al.* (1956) was maintained and used in the same manner as HeLa cells.

Culturing of specimens. Specimens were transferred immediately to vials containing 2 ml of Eagle's medium without glutamine and serum. Usually the specimens were refrigerated *ad interim* and inoculated into the cell cultures within a few hours; otherwise they were stored at −55°C and inoculated within 24 hr. Two tubes of each type of cell culture were inoculated, each with 0·2 ml of the specimen, and another two were inoculated with 0·2 ml of Earle's balanced salt solution. After 2–3 days' incubation the cultures were examined for evidence of viral activity. Even if none was observed, a 0·2 ml portion of the culture fluid was transferred to a tube of fresh cell culture. Two such passages were deemed sufficient to determine the presence or absence of a virus (HABEL, K., personal communication).

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Identification of virus

Herpetic cytopathic effect (CPE). Isolations of herpes simplex virus were identified by their characteristic CPE and its prevention by herpes antiserum. The CPE in rabbit kidney cells conformed closely to the descriptions of SOSA-MARTINEZ, GUTIERREZ-VILLEGAS and SOSA (1955) and SCOTT and MCLEOD (1959), i.e. some infected cells ballooned and developed a granular appearance whereas others appeared to form multinucleate giant cells. Some infected HeLa cells coalesced to form syncytial giant cells whereas others agglutinated into piles of rounded ballooned cells (GRAY, TOKUMARU and SCOTT, 1958; SCOTT and MCLEOD, 1959). In both cell systems, the CPE was accompanied by a general disintegration of the cellular sheet. The herpetic CPE in cultured human skin cells seems not to have been described. We found it to be identical with that seen in HeLa cell cultures (Figs. 1–4). Monkey kidney cells, which develop no CPE from herpes simplex virus, remained unaffected in the present experiments by the specimens presumed to contain this virus. That the cultures of monkey cells were adequately exposed was demonstrated by the CPE of their supernatants on susceptible cells. These observations substantiated the identity of our isolates as herpes simplex virus. Examination of infected cells for

TABLE I. NEUTRALIZATION OF HERPES SIMPLEX VIRUS BY ANTISERUM IN RABBIT KIDNEY CELL CULTURES

Virus		Rabbit serum	CPE from 50 $TCID_{50}$ of virus +serum diluted*						
Strain	Titre		1:4	1:8	1:16	1:32	1:64	1:128	1:256
11123 (stock)	$10^{7\cdot2}$†	Normal	++++						
		Anti-11123‡	–	–	–	–	–	–	+
11125	$10^{6\cdot7}$	Normal	++++						
		Anti-11123	–	–	–	–	–	+++	++++
11126	$10^{7\cdot3}$	Normal	++++						
		Anti-11123	–	–	–	–	–	+	++++
11129	$10^{6\cdot7}$	Normal	++++						
		Anti-11123	–	–	–	–	–	+	++++
11130	$10^{7\cdot3}$	Normal	++++						
		Anti-11123	–	–	–	–	–	–	+
11133	$10^{7\cdot2}$	Normal	++++						
		Anti-11123	–	–	–	–	–	–	++

* $TCID_{50}$ of virus in 0·1 ml+0·1 ml of diluted serum. Extent of CPE rated arbitrarily.

† $TCID_{50}$ per ml after three passages in rabbit kidney cell culture.

‡ During a period of 3 weeks, each rabbit received a series of sixteen intra-abdominal injections of 10^4 $TCID_{50}$ (1 ml vol) of cell-free supernatants of cultures of strain 11123 in HeLa cells. Blood for serum was withdrawn 2 weeks after the last injection.

Type A intranuclear inclusions was not done routinely; however, some infected cultures were stained (Giemsa) and revealed the inclusion bodies typical of herpes simplex virus. After one subculture, each newly isolated strain of herpes virus was cultured in a number of tubes of rabbit kidney cells. The culture fluids were pooled, titred to determine the average tissue culture infective dose ($TCID_{50}$) (LENNETTE, 1959), and stored at −55°C. For a neutralization test, portions of this stock virus, diluted in PBS to contain 500 $TCID_{50}$ per ml, were mixed with equal volumes of serial twofold dilutions of inactivated sera diluted in PBS. After an hour at room temperature, 0·2 ml portions of each mixture were inoculated into each of three tubes of rabbit kidney cell culture. These tubes were examined for herpetic CPE after incubation for an additional 4–5 days. Typical results are presented in Table 1.

Other tests for viruses. Two procedures were used to seek evidence of viral activity in cultures showing no CPE. The haemadsorption test with guinea pig erythrocytes was carried out as described by SHELOKOV, VOGEL and CHI (1958) for detecting influenza virus. Haemadsorption was demonstrated readily in cultures of influenza virus in monkey kidney cells. The second procedure was freezing, thawing, and subculture to test for liberated virus. The tubes showing no CPE were rotated in a mixture of solid carbon dioxide and Methyl Cellosolve (2-methoxy-ethanol) until the contents froze, then immersed in water at 37°C to thaw. Three repetitions of this procedure completely disrupted the cytoplasmic structure of the cells and should have sufficed to release any intracellular virus. The cell debris was removed by centrifugation and 0·2 ml portions of the supernatants were inoculated into cultures of all four cell types, which were incubated and examined for CPE after 2–3 days.

RESULTS

Eleven patients with recurrent herpes labialis and thirty-one with recurrent aphthous ulcerations were studied over a period of 18 months. Of twenty-five herpetic lesions cultured, thirteen yielded herpes simplex virus, which was serially

TABLE 2. VIRAL ISOLATIONS IN CELL CULTURES FROM RECURRENT FEVER BLISTERS AND CANKER SORES

	Recurrent fever blisters	Recurrent canker sores
No. of patients	11	31
No. of lesions cultured	25	100
Mean no. lesions cultured per patient*	2·3	3·2
No. cell cultures positive		
HeLa	13/25†	0/100
Human skin	13/25	0/100
Rabbit kidney	13/25	0/100
Monkey kidney	0/25	0/100
Percent specimens positive	52	0
Percent patients positive	64	0

* Only one lesion cultured per patient per visit.
† Number of isolations of herpes simplex virus per number of lesions cultured.

transferable in cultures of HeLa, human skin, and rabbit kidney cells; none of the other twelve gave evidence of any viral activity. None of these twenty-five specimens affected monkey kidney cells. The newly isolated strains were derived from seven patients. All were neutralized by antiserum to a stock strain. The results of representative neutralization tests are summarized in Table 1.

None of the 100 recurrent aphthous ulcerations cultured gave evidence of any viral activity, i.e. positive haemadsorption tests or CPE in primary cultures and subcultures with the four cell types used. These results are summarized in Table 2.

DISCUSSION

Although the diseases considered in the present investigation present clinical similarities, those caused by herpes simplex virus (primary acute herpetic gingivostomatitis and recurrent herpes labialis) have distinguishing characteristics. The virus can be isolated readily by inoculation of specimens in the scarified corneas of rabbits, in embryonated eggs, in mice, and in cultures of rabbit kidney, HeLa, and human skin cells. Human transfer studies have demonstrated the aetiological relationship of recurrent herpes labialis to primary acute herpetic gingivostomatitis (BLACK, 1942). Antibody to herpes virus is absent at the onset of the primary infection, increases rapidly to high titre during convalescence, and persists throughout life, often at a high level (BURNET and LUSH, 1939). All patients with recurrent herpes labialis have herpes antibodies, which usually do not increase concurrently with new lesions (DASCOMB *et al.*, 1955). (See DRISCOLL *et al.*, 1959, for a review of the mechanisms of chronic virus infections). On the other hand, the fact that as many as a third of patients with recurrent aphthous ulcerations lack herpes antibody (STARK *et al.*, 1954) makes it *a priori* most unlikely that these lesions result from herpes simplex virus. Furthermore, this virus has been isolated so rarely from such lesions that its presence must be regarded as adventitious.

The present results substantiate previous investigations, in that herpes simplex virus was readily isolated from lesions clinically diagnosed as herpes labialis but never from recurrent aphthae. More important and generally significant was the fact that specimens from the latter type of lesion, taken from the same patients on several occasions and at various stages of development, gave no evidence of any other kind of viral activity in four types of mammalian cell cultures known to be susceptible to a wide variety of viruses.

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FIGS. 1–4. Cytopathic effect of herpes simplex virus in mammalian cell cultures. Unstained. ×120.

FIG. 1. HeLa cells, uninfected.
FIG. 2. HeLa cells, 60 hr after inoculation of 10^4 $TCID_{50}$ of virus per ml.
FIG. 3. Human skin cells, uninfected.
FIG. 4. Human skin cells, 60 hr after inoculation of 10^4 $TCID_{50}$ of virus per ml.

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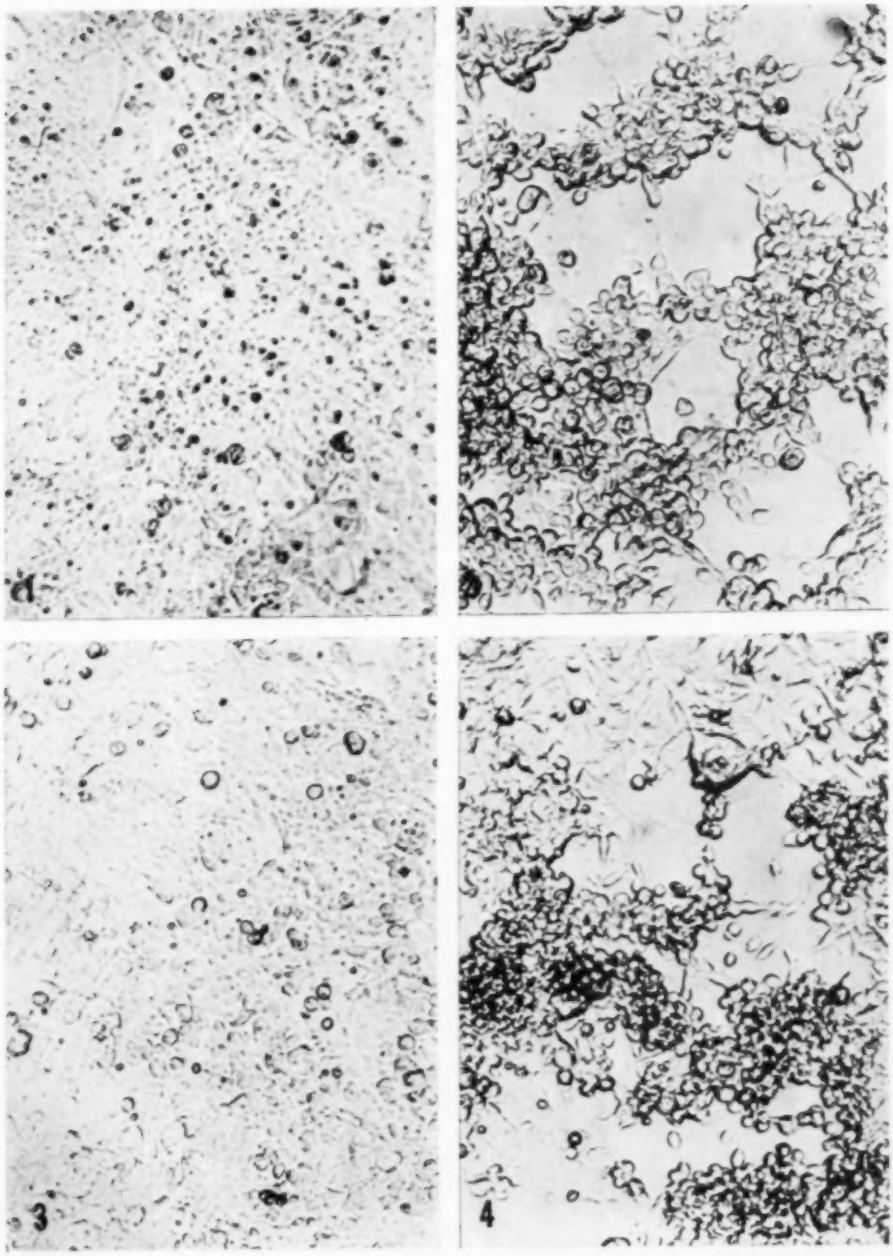

PLATE 1

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THE MYCOTIC FLORA OF THE HEALTHY MOUTH

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Abstract—From the plaques on the necks of posterior non-carious teeth positive cultures of fungi were obtained with frequencies varying between 20·8 and 58·3 per cent in different groups of juvenile persons. A higher frequency of positive cultures was found in females than in males, and more during the summer than during the winter. The fungus most often recovered was *Candida albicans*.

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THE mycotic flora of the oral cavity has been frequently investigated in the past. Recently, clinical interest in fungi has increased because the wide use of antibiotics against bacteria has sometimes led to a preponderance of fungi in the mixed flora. Furthermore some fungi, mostly yeasts and yeast-like forms, "normally" non-pathogenic—i.e. in the undisturbed microbial equilibrium in the mouth—are said to acquire pathogenic properties (SELIGMANN, 1953; HUPPERT, CAZIN and SMITH, 1955). Thus, research concerning the portals of entry into the host becomes relevant. In this connexion the nasal and oral cavities, being entrances to the respiratory and digestive tracts, as well as the skin, must be considered.

In the oral cavity several fungi have been demonstrated, particularly budding forms representing various strains of *Candida*. According to FEJÉR *et al.* (1957), RAVAUT and RABEAU (1936) grew yeast and yeast-like organisms in approximately 80 per cent of cultures from healthy mouths. EPSTEIN (1931) found the incidence of *Candida* in the lip commissures to be 38·5–54 per cent of children up to 6 years old, but only 10 per cent of adults. *Candida albicans* was demonstrated by MÜLHENS (1955) in 5 per cent of persons with caries-free dentitions, but in 20 per cent of persons with non-treated caries. Immediately following antibiotic therapy the frequency figure increased from 5 to 80 per cent.

These results emphasize the wide variability of mycological findings even for a single type of fungus. Such differences of findings are attributable to the natural variation of the mycotic flora in different sites and at different times. Therefore an attempt was made to examine the "normal" mycotic flora under standard natural conditions.

MATERIAL AND METHODS

Inocula for cultures were taken from the plaques adherent to the necks of non-carious posterior teeth in juveniles free from oral disease, including gingivitis, and with no oral manifestations of general disease. In preliminary sampling, a more

consistent flora was demonstrated at this site than on the mucous membrane surfaces of cheeks, tongue, and even gum. These sites are more exposed to friction during movements in speech, mastication, etc., and are changed by the flow of saliva, while at the necks of the posterior teeth, plaques, indicative of relative stagnation, are constantly present. The plaque, containing food remnants such as bread, other farinaceous material, sugar and potatoes, provides the nutrients necessary for fungi to thrive.

Over a period of 9 months samples were collected from 154 subjects undergoing routine half-yearly inspection by the School Dental Service at our Department. The subjects comprised eighty-one females and seventy-three males between the ages of 7 and 20, the vast majority being aged 13–19 years. The examinees had carried out their customary morning oral hygiene and had their usual breakfast. Samples were collected between 8 and 10 a.m. but not less than 1 and not more than 4 hr after eating.

From the debris at the necks of lower premolars or molars inocula were collected with a sterile platinum loop. Material from the same site was spread over two kinds of solid media, namely Sabouraud's glucose-peptone agar, and "mash"-agar. The latter was prepared according to the description of URI, SZABÓ and OLÁH (1952) by soaking dried barley and adding 2–3 per cent agar. The medium contains various saccharides, especially maltose, and low concentrations of various amino acids. Several years of practical experience have shown this medium to be as satisfactory for the growth of fungi as more elaborate ones. The plates were incubated at 30°C for 74 days. The use of two media acted as a safeguard since in some cases growth was obtained on one medium but not on the other. In some instances, media were incubated at 37°C also.

RESULTS

Differences were noted in the frequency of positive cultures between males and females on the one hand, and between summer and winter months on the other hand (Table 1). In Table 1 the number of positive cultures is shown with regard to the media used. In Table 2 a list of fungi recovered from the plates is shown.

TABLE 1. THE FREQUENCY OF POSITIVE FUNGUS GROWTH

Period	Sex	No.	Growth of fungi				Positive fungus growth		Bacterial growth on		
			Negative	Positive			Total	%	Sabouraud	"Mash"	Both
				Sabouraud	"Mash"	Both					
Winter months	F	33	22	5	3	3	11	33·3±8·0	–	8	1
	M	24	19	–	2	3	5	20·8±8·2	–	9	1
Summer months	F	48	20	8	6	14	28	58·3±7·1	5	5	10
	M	49	32	4	4	9	17	34·6±6·8	6	4	9

TABLE 2. FUNGI RECOVERED FROM SOLID CULTURE MEDIA

Candida albicans	50
Penicillium species	10
Scopulariopsis brevicularis	3
Geotrichum asteroides	3
Hormodendrum compactum	1
Aspergillus species	1
Hemispora stellata	1
Double fungus infection	8
Fungus + bacterial growth	9

DISCUSSION

The higher frequency in females than in males is striking. This sex difference is notable in both the winter and summer periods. The summer sex difference is statistically significant at 5·0 per cent level ($D = 23{\cdot}7 \pm 9{\cdot}8$ per cent), but the winter sex difference is not. Our findings agreed with those of TODD (1937) who found a sex difference in the frequency of *Candida albicans* (30·4 per cent positive cultures in females but only 15·7 per cent in males). A satisfactory explanation cannot at present be given for this sex difference. It is noteworthy, however, that positive fungus findings were recorded in the vaginal flora in 36 per cent of cases by DÖDERLEIN (1937) (cited by GOTTRON *et al.*, 1958) and in 37 per cent by JOHNSON and MAYNE (1948), although a direct connexion between vaginal and oral mycotic floras must not be assumed. A possible explanation of the sex differences found in respect of the oral mycotic flora may lie in the well-known higher prevalence of dental caries in females. This explanation is consistent with the aforementioned findings of MÜLHENS who recorded a fourfold greater frequency of positive findings in persons with carious teeth as compared with caries-free ones.

The difference in the frequency of positive fungus findings during the winter and summer months is obvious. In the summer, the frequencies of positive cultures are approximately 15–25 per cent higher. The seasonal difference is statistically significant at the 5 per cent level in females ($D = 25{\cdot}0 \pm 10{\cdot}8$ per cent) but not in the males. Since 70 per cent of the positive findings are due to *Candida albicans*, the seasonal difference may be explicable on the following grounds: many references in the literature show that *Candida* strains occur in the soil of kitchen gardens, orchards, and farmyards. They also occur commonly on raw fruits and vegetables. Not only are those foods consumed mainly in the summer but at this time the air is often heavily polluted with moulds. In addition, the Hungarian summer diet contains higher amounts of fermentable carbohydrate, and oral remnants of such foods form an excellent medium for the growth of fungi. This combination of factors may contribute to the increased incidence of fungi during the summer.

The few data of positive bacterial growth are mentioned for the sake of completeness. In spite of having used selective media for fungus growth, bacteria sometimes grow on Sabouraud's as well as on "mash"-agar. Bacterial growth was observed mostly in instances when no growth of fungi was obtained. Two kinds of bacteria

were recovered from the plates: *Bacillus subtilis* and *Sarcina lutea*. Streptococci and staphylococci or other cocci were not grown in any instance.

According to Table 2, *Candida albicans* was the most frequent mould in the plaque on premolars and molars, amounting to more than 70 per cent of positive cultures. *C. albicans* is followed by different Penicillium species, while other fungi were demonstrated in rather rare instances.

Our findings indicate that the oral cavity is a primary portal of entry for *Oidomycetes*. Through the mouth they enter the digestive tract, and perhaps the lungs and other internal organs. Their change from a saprophytic to a parasitic mode of existence is promoted no doubt by the wide and indiscriminate use of anti-bacterial antibiotics. Our findings do not invalidate the possibility of contact infection of the integument (skin, nails, etc.) which may play a role in the formation of deep infections.

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HOMOTRANSPLANTATION OF TOOTH BUDS

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Abstract—Freshly dissected molar tooth buds taken from 19-day rat foetuses were transplanted to adult male hosts. The sites of implantation were under the skin and under the oral mucosa. These grafts were rejected. Tooth buds of the same stage were cultured in a medium containing the host's serum for 12 days, and then transplanted to adult male hosts. None of these grafts was rejected. The development was slower than *in vivo*. The enamel failed to mature. The dentine calcified and root formation followed the normal pattern.

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INTRODUCTION

THIS study was designed to test an organ culture method as a tooth bank. Since the investigation depended upon transplantation of tooth buds, several facets of homotransplantation entered into the project.

The study established that rat tooth buds stored *in vitro* retain their viability and develop as grafts when replanted. Furthermore, the cultured tooth buds were not rejected by the host; although the uncultured tooth buds were.

MATERIALS AND METHODS

All tooth bud grafts were dissected from 19-day foetuses of an inbred Sherman albino strain of rats (LEFKOWITZ and MARDFIN, 1954). The hosts were adult male rats of the same strain. The host animals were not used as sires.

A previous study (LEFKOWITZ, BODECKER and MARDFIN, 1953) provided the control sections of a 19-day rat foetus first molar (Fig. 1). This established the stage of development at the time of implanting or explanting.

Twenty-seven freshly dissected tooth buds were implanted in fifteen host animals. Twelve of these received two grafts at the same time, one implanted beneath the dorsal skin, the other in the submucosa of the maxillary notch anterior to the first molar. Three host animals received one submucous graft each. Of this group, eight submucous and five subcutaneous grafts were recovered for histologic examination (Table 1).

Fifteen tooth buds were cultured *in vitro* before implanting (LEFKOWITZ and SWAYNE, 1958). The medium contained 10 per cent serum previously derived from the host, and the period of cultivation was 12 days. Fig. 2 is a section of a 19-day rat molar tooth bud which had been cultured *in vitro* for 12 days. This control demonstrates the stage of development at the time of implanting.

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Seven host rats received two cultured grafts, one implanted in the submucosa and one in the subcutaneous tissue at the same time. The other host received only one graft implanted in the submucosa. Of this group, two grafts implanted subcutaneously and six submucously were recovered for histologic examination (Table 1).

TABLE 1. FATE OF FORTY-TWO TRANSPLANTED TOOTH BUDS

	Submucous uncultured	Grafts recovered	Subcutaneous uncultured	Grafts recovered	Submucous cultured	Grafts recovered	Subcutaneous cultured	Grafts recovered
2 hr	1	0	1	0				
3 days	1	1	1	1				
1 week	3	2	2	1	3	2	2	1
2 weeks	3	2	2	1	3	2	4	1
3 weeks	3	2	2	2				
4 weeks	1	1	1	0				
6 weeks	2	0	2	0	2	2	1	0
13 weeks	1	0	1	0				
Total	15	8	12	5	8	6	7	2

At the time of implanting, the graft was dissected from the coagulum and transferred to a balanced salt solution containing 10 per cent host serum until the sites were prepared.

SURGICAL TECHNIQUE

The subcutaneous grafts were implanted in an incision made between the scapulae. Some were placed in the reticular layer, others in the panniculus adiposus, depending on the depth of the incision. The graft was placed into the open wound by a pipette and then the wound was sutured.

In the submucous area, a semi-lunar incision was made in the maxillary notch. The mucous membrane was reflected and the tooth bud placed against the exposed bone by means of a pipette. The flap was then sutured in place.

Block specimens were taken at 2 hr, 3 days and then weekly. They were decalcified and processed for histologic study. The sections were stained in haematoxylin and eosin.

RESULTS

Subcutaneous grafts (*uncultured*)

Three days after implantation the avascular graft increased in size but the elements did not advance in development. There was a blood clot in the area of the incision (Fig. 3).

At the end of 1 week, the graft showed a marked increase in development and size (Fig. 4). The papilla became vascularized, the odontoblasts and ameloblasts had differentiated and a layer of dentinoid, calcified dentine and matrix enamel had

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formed. The tooth bud was encapsulated in the dental sac. It was in the dental sac, however, that the first evidence of rejection appeared, in the form of a dense infiltration of lymphocyte-like (lymphoid) cells.

The 2-week sections showed further growth and development (Fig. 5). The surgical injury, faintly evident after 1 week, had been fully repaired. The lymphoid infiltration in the dental sac increased but had not yet invaded the dental papilla. The vascular dental papilla, the odontoblasts and the enamel organ consisting of the ameloblast layer and stratum intermedium remained normal.

At 3 weeks, the dental papilla had degenerated and lymphoid cells appeared in this area. The enamel organ persisted as a layer of degenerating squamous epithelium, whilst the matrix enamel and dentine were unaffected. The histologic picture showed the delayed rejection of the graft by the host (Fig. 6).

Submucous grafts (uncultured)

Three days after implanting, the tooth bud graft, placed beneath the oral mucosa, was similar to that in the subcutaneous implant (Fig. 3). Since no receptacle had been prepared in the bone, the tooth bud was deformed by the pressure of the oral mucosa.

Specimens taken at 1 week also showed the deformation of the graft. Nevertheless, the tooth bud increased in size. Although ameloblasts, odontoblasts and a thin layer of dentinoid had formed (Fig. 7), the development lagged behind the corresponding subcutaneous graft. The papilla had not become vascularized, and there was a mild lymphoid infiltration in the dental sac.

The 2-week specimens showed the graft in contact with the bone (Fig. 8). A thin distorted layer of dentinoid, dentine and matrix enamel had formed. In no way did the morphology of the graft resemble a developing rat molar. It was devoid of cusp formation at the dentino-enamel junction. There was a dense lymphoid infiltration peripheral to the enamel epithelium. The central area of the dental papilla was occupied by bone which was ankylosed to the dentine. The remainder of the papilla contained few cells. No resorption of dentine occurred. The enamel epithelium changed to the squamous form (Fig. 9). The thin incomplete layer of matrix enamel, tubular dentine and predentine demonstrated that normal formation had been arrested. The typical odontoblasts were absent although some degenerated odontoblasts could still be recognized. Most of the papillary area was occupied by necrotic pulp cells, fibroblasts, lymphoid cells and capillaries. Bone formation within the dental papilla was still active as established by the osteoblastic layer.

Graft specimens taken at 3 weeks showed resorption of the formed matrix enamel and dentine (Fig. 10) and none of the original soft tissues of the graft remained. With the exception of one small zone of bone resorption, osteogenesis was active in the papillary area. At this time, lymphoid infiltration was no longer present, but there was resorption of the dentine and enamel which was formed after implanting the graft (Fig. 11).

This is the histologic picture of the rejection of a tooth bud graft by the host. Not only is there necrosis of the graft cells but the formed products are resorbed,

however late. In the subdermis, the process of rejection is slower than in the submucous area. This part of the study established the time and pattern of rejection.

Subcutaneous grafts (cultured)

One week after implanting, the formative cells, dentine and matrix enamel developed normally. Although the morphology of the graft appeared altered because of the plane of the section, there was evidence of cusp formation. The dental sac contained no infiltrative cells (Fig. 12) which, if present, would denote an early sign of rejection as in the uncultured grafts.

The 2-week specimen showed a slight advance in development. The formative cells were normal and the papilla and dental sac were vascular. Evidence of the rejection phenomenon was notably absent (Fig. 13).

Submucous grafts (cultured)

One week after implanting a cultured tooth bud in the submucosa of the maxillary notch, the graft showed rapid growth and development. The dentino-enamel outline, the formation of dentine and matrix enamel and soft tissue components of the tooth bud demonstrated growth and development. The dental papilla was also vascular (Fig. 14).

A 2-week specimen showed further development and formation. Unfortunately, the section was cut transversely at an oblique angle and demonstrated essentially the formation of dentine, dentinoid and matrix enamel. The papilla was vascular and ameloblasts and odontoblasts normal. There was no evidence of rejection (Fig. 15).

Two cultured tooth buds implanted submucously for 6 weeks showed no evidence of rejection. The development of the graft, though retarded in contrast to normal development, had advanced to root formation. The papilla (pulp) was normally vascular and bordered by odontoblasts. Dentinoid and a relatively thin layer of dentine extended into the forming root. The crown was covered with a layer of matrix enamel. A thin layer of cementum covered the gingival third of the root. The presence of Hertwig's sheath with its bulbous growth centre indicated that root formation was in progress. The entire graft, surrounded by the dental sac, rested in a bony crypt. The inner surface of the crypt showed resorption, indicating continuing growth. There was no evidence of rejection, ankylosis or metaplasia (Fig. 16).

The major difference between the 6-week graft and the normal odontogenic pattern was in enamel maturation. In all tooth bud grafts, the enamel remained in the matrix state.

DISCUSSION

The one common denominator of homotransplantation experiments is failure. At best, save the isolated instances of success, the laws of genetics (SNELL, 1957) and immunology (MEDAWAR, 1959) control the fate of most grafts. Other influences such as size of the graft, site of planting and species of experimental animal also influence the result.

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What constitutes failure? In this study there were two kinds of failure; those grafts which were not found after resection and those which showed evidence of rejection.

Failure to recover the graft should not be considered evidence of rejection. The surgical procedure used to implant a 0·5 mm elipsoidal organ was of doubtful reliability. Even in those instances where the surgical wound established the site of implanting only about half of the grafts were found on sectioning. This was most dramatic in the 1-week specimens where the injured site was evident and the graft was not found.

The other possibility is a new pattern of immediate rejection. This is inconceivable since two of the 2-hr grafts were not found in the resected tissue (Table 1).

If we consider only those specimens in which the tooth bud grafts were recovered, the patterns of rejection and acceptance become clear. All uncultured grafts were rejected. The pattern of lymphoid infiltration and the timing of regression were similar in every case. On the other hand, the cultured grafts did not evoke the lymphoid infiltration and showed no evidence of cellular necrosis or resorption of the formed tissues. They were planted as avascular organs and were vascularized by the host.

Similar results have been reported for cultured endocrine grafts (GAILLARD, 1954; LUX, HIGGINS and MANN, 1937; MARFINOVICH, 1956; STONE, OWINGS and GEY, 1934) although, as MEDAWAR (1959) puts it, "They survive for reasons unknown". There is reason to believe that embryonic homografts survive for longer periods than adult grafts (CANNON, 1957; DOUGLAS *et al.*, 1954). Still, both cultured grafts (LUX *et al.*, 1937) and embryonic grafts (HELSINGEN and HELSINGEN, 1957; ROSEN and SILVERS, 1956) have been shown to lack histo-compatability.

In this study, there are two significant factors which cannot be overlooked. Donors and hosts were from an inbred strain. Therefore we may consider them isologous and the experiment, in essence, was autotransplantation. Furthermore the hosts were male animals. It is known that in certain strains of mice, female hosts reject skin grafts from male donors (EICHWALD and SILMSER, 1955).

Despite the isologous pattern and the sex influence these results and other isolated "takes" suggest another, as yet unknown, factor. Can it be an accident that all recovered uncultured grafts were rejected, or can we assume that the uncultured grafts were implanted in heterogenous hosts? Conversely, can we assume that all cultured grafts were implanted in isologous hosts? Indeed, this study does not provide the answers.

The age of the graft differed in the two experiments. The uncultured graft was from a 19-day foetus. The "*in vivo*" age of the cultured graft was 21 days. If we were to ignore other factors, the age of the donor tissue might seem important. However, in comparison with the established factors of histocompatibility genes and immunologic laws relating to the rejection of embryonic transplants, age specificity is insignificant (BAXTER and GOLDSTEIN, 1958).

A few dental observations deserve mention. The uncultured tooth bud grafts increased in size, formed matrix enamel and dentine before the lymphoid cells

appeared in the dental sac. Despite this, the growth potential of the graft continued and the amount of formed tissue increased quantitatively. The grafts, avascular when implanted were vascularized within the first week.

The formed matrix enamel and dentine were eventually resorbed in the uncultured tooth buds. The acellular matrix enamel and dentine which, after necrosis of the odontoblasts, must be considered devoid of odontoblastic processes were removed by resorption.

Of particular interest is the bone formation in the area of the dental papillae in uncultured grafts. This occurred after lysis of the cells of the papilla only when the graft was implanted close to the periosteum.

The vascular pattern of both cultured and uncultured grafts was initiated at the same time. After the foreign cells degenerated, the blood supply to the transplant failed in the uncultured tooth bud grafts, whilst in the cultured grafts the vascularity persisted.

The relationship of the vascularity to rejection or acceptance of the graft is vague and contradictory (EICHWALD, 1959). EICHWALD, discussing tumour grafts, stated "There is evidence that a graft does not elicit a strong immune response unless it is vascularized by the host. There is also evidence that such a response is not readily transmitted to a graft unless it is vascularized." In this study, both rejected and accepted grafts showed early vascularization.

In all cases, the enamel failed to mature, even 6 weeks after implanting. It also fails to mature in tissue culture (LEFKOWITZ and SWAYNE, 1958). Normally the enamel of the first molar of the rat matures 16 days after birth (UOHARA, 1959). This finding suggests a worthy area of biochemical and histochemical investigation of enamel maturation.

Tooth buds, partially formed and fully formed teeth have been used as auto-, homo- and heterografts with reported success in experimental animals and human beings (AGNEW and FONG, 1956; CSEREPFALI, 1959; FLEMING, 1956, 1959; HAHN, 1941; HUGGINS, MCCARROLL and DAHLBERG, 1934; HUNTER, 1771; MYERS and FLANAGAN, 1958). In no report was the rejection phenomenon described. Success depended on continued formation of the roots, periodontal attachment and on pulp viability. Throughout the reports, there is a strong suggestion that the laws which govern transplantation do not apply to teeth or their anlage. Perhaps dental grafts, like endocrine grafts, "survive for reasons unknown". What is significant is that there is a rejection pattern as herein described.

Should homotransplantation become successful, a tooth bank must be developed. Organ culture has much to offer as a "bank" method; the most significant point is that a tissue or organ demonstrates viability (PERRY *et al.*, 1956).

Transplantation of tooth buds stored in a balanced salt solution or merthiolate at low temperatures was unsuccessful (CSEREPFALI, 1959), as were transplantations of frozen teeth (PAFFORD, 1956; WAITE, 1956). Frozen tooth buds fared better (GERSTNER and BUTCHER, 1958, 1959; YOSHIOKA and GONZALES, 1959). However, the animal experimentation in inbred strains of particular species (ADAMS, PATT and LUTZ, 1956) cannot solve the rejection problem.

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Were it not for the established immunologic and genetic rules which govern histocompatibility, it would appear that the culture technique altered the antigenic properties of the graft. Only repetition of this study with different strains for the donor and host will establish the possibility of despeciation of tooth buds by culture methods.

In summary, the tooth bud culture method offers a satisfactory approach to the tooth bank. By reducing the temperature, as suggested by CARREL (1912), the rate of development could be retarded. This study established that tooth bud culture passed the critical test of transplantation in rats.

The results of this investigation suggest that cultured tooth bud grafts, like some endocrine glands, are exceptions to the usual pattern of graft rejection. The cultured tooth bud should provide an interesting and useful organ for transplantation investigation. Future studies should test the immunology and genetic histocompatability of cultured tooth buds. This should establish whether the tooth bud is an exception or if the culture technique despeciates a tooth bud graft.

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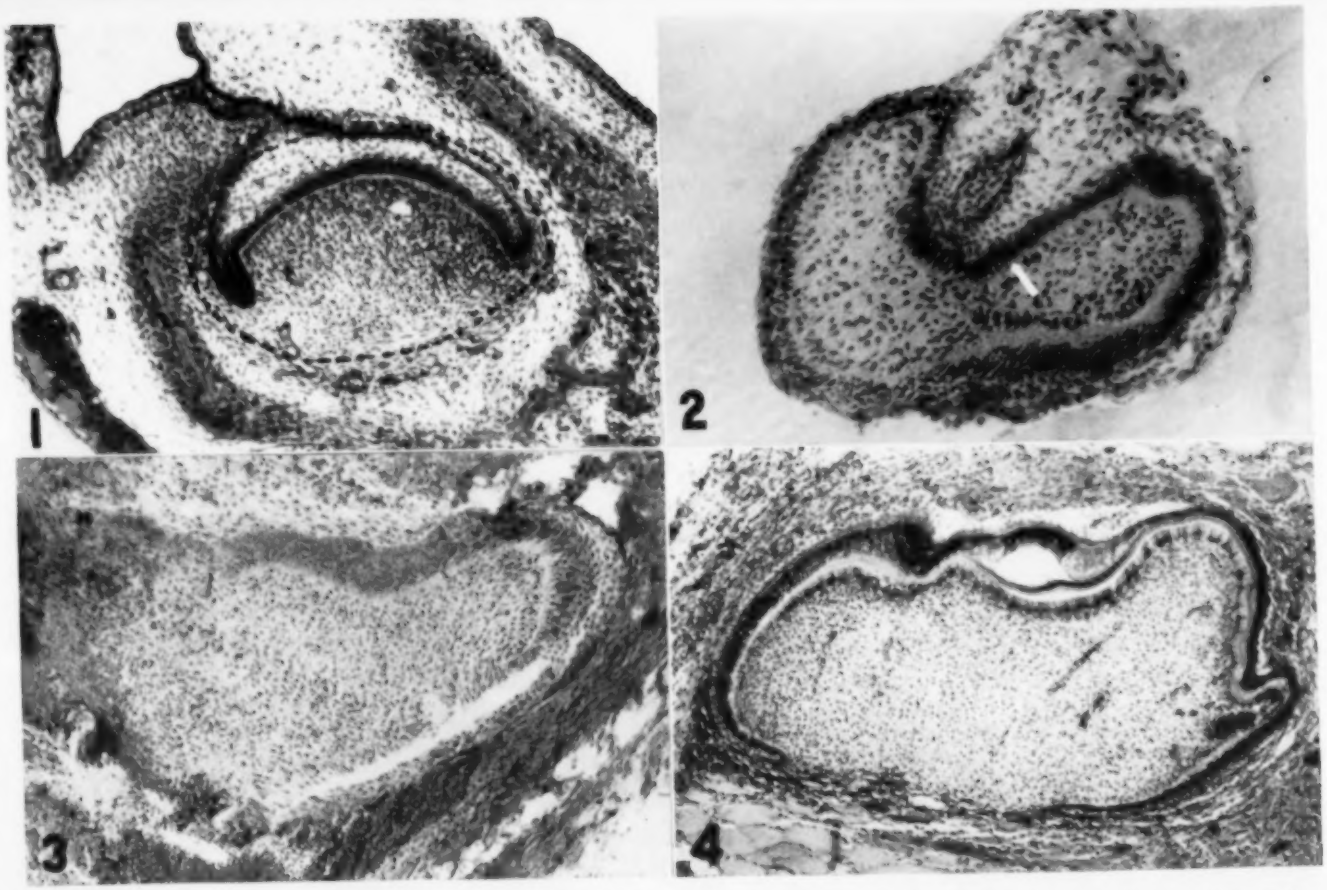

FIG. 1. *In situ* first molar tooth bud of a 19-day rat foetus. Dotted line indicates dissected tooth bud. Haematoxylin and eosin. ×35.

FIG. 2. Tooth bud of 19-day rat foetus cultured for 12 days. Note the advanced development. Arrow points to formation of dentinoid. Haematoxylin and eosin. ×50.

FIG. 3. Uncultured tooth bud 3 days after implantation subcutaneously. Haemorrhage at top resulted from surgery. Haematoxylin and eosin. ×30.

FIG. 4. Uncultured tooth bud 1 week after implantation subcutaneously. All elements developed normally, and the papilla became vascularized. Note the lymphoid infiltration in the dental sac. Haematoxylin and eosin. ×30.

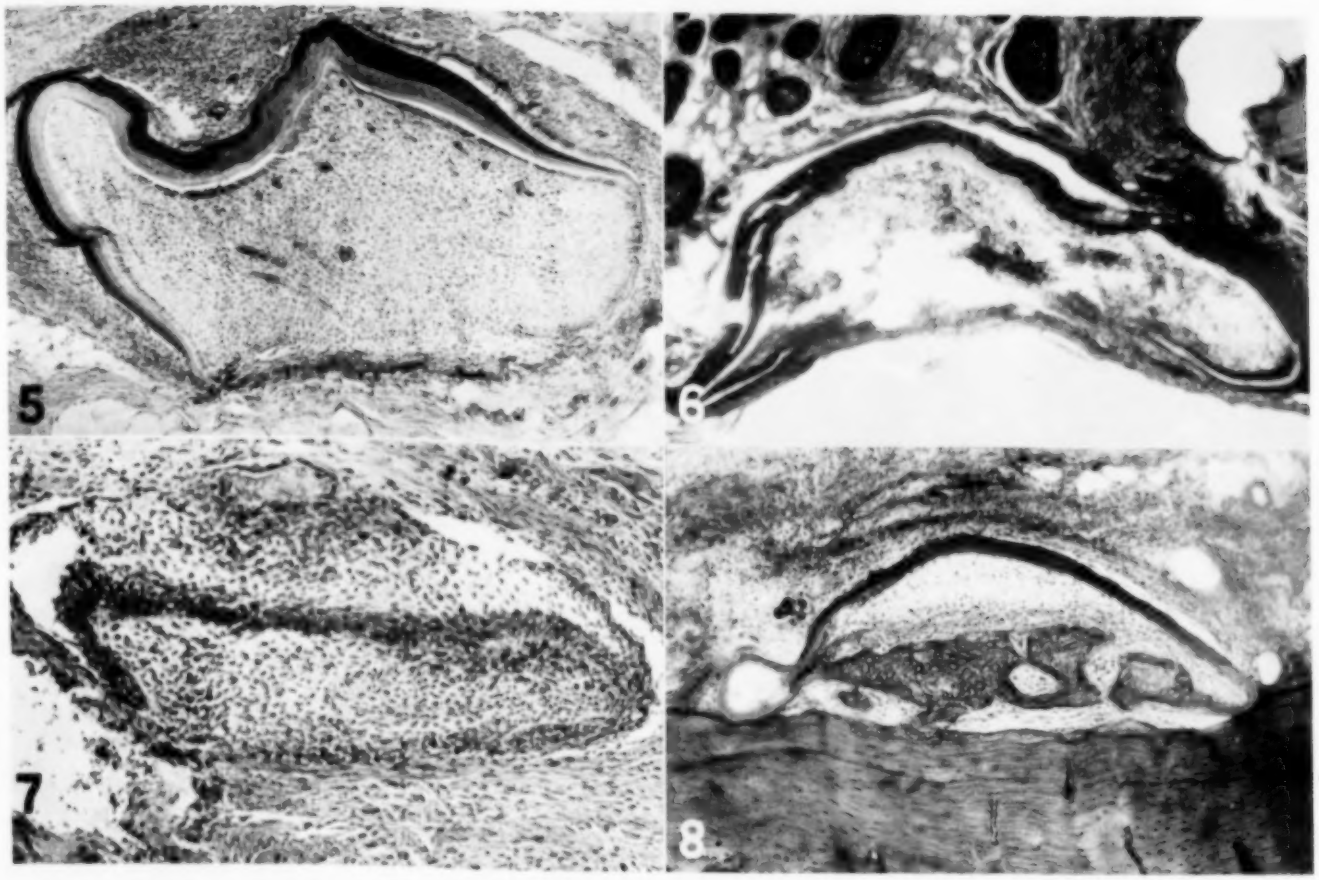

FIG. 5. Uncultured tooth bud 2 weeks after implantation subcutaneously. Note the increased lymphoid infiltration in the dental sac. Haematoxylin and eosin. ×30.

FIG. 6. Uncultured tooth bud 3 weeks after implantation subcutaneously. The cells of the papilla are necrotic. The enamel epithelium is altered to a squamous epithelium. Enamel matrix and dentine are unaffected. Haematoxylin and eosin. ×30.

FIG. 7. Uncultured tooth bud 1 week after implantation beneath the oral mucosa. Note the flat appearance and lack of dentine cusp development. Haematoxylin and eosin. ×50.

FIG. 8. Uncultured tooth bud 2 weeks after implantation beneath the oral mucosa. A bony mass occupies the papillary area. The morophology is similar to Fig. 7. Note the dense lymphoid infiltration peripheral to the graft. Haematoxylin and eosin. ×42.

PLATE 2

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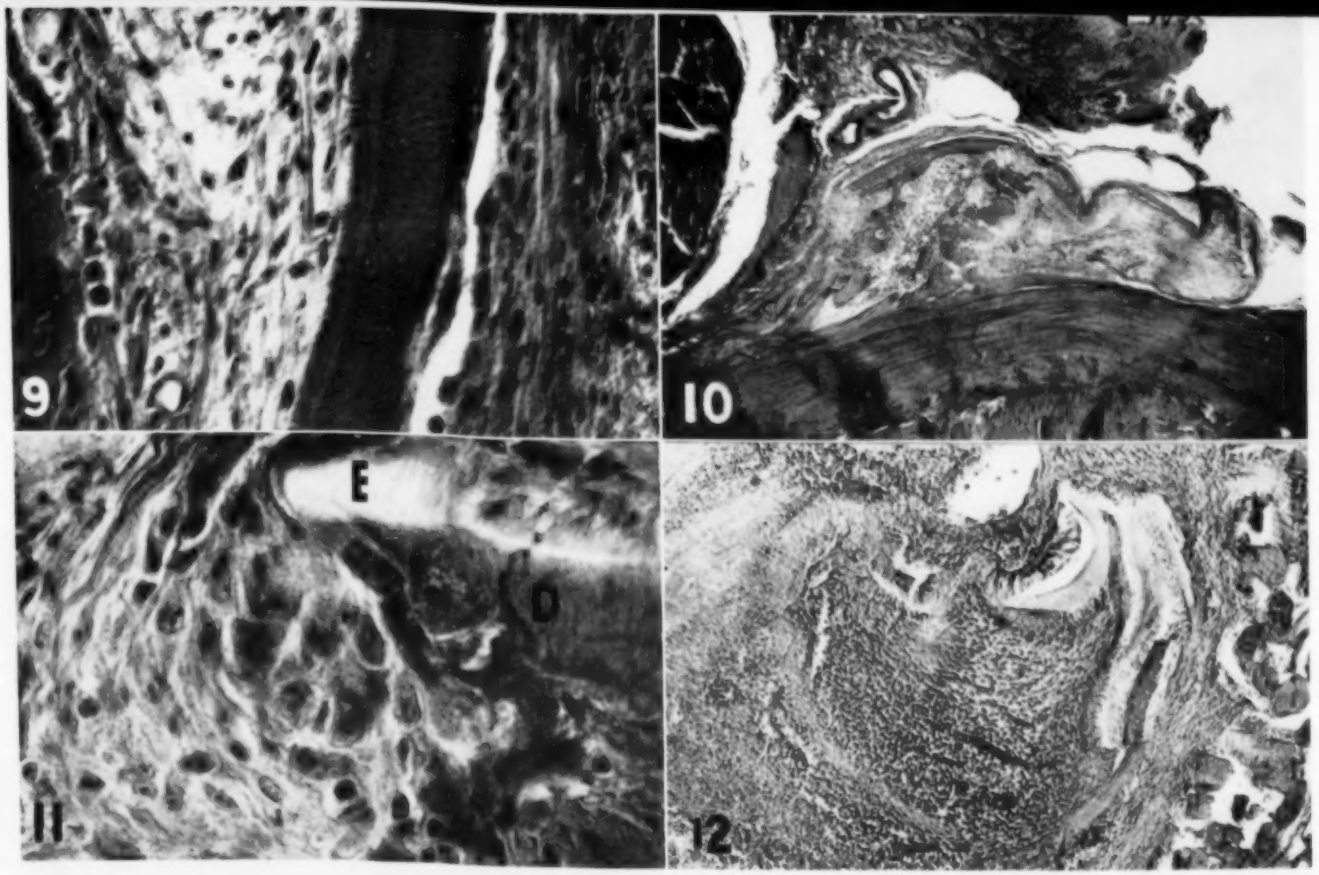

FIG. 9. Higher magnification of area of Fig. 8. The enamel epithelium has become altered to a squamous pattern. There is a thin layer of matrix enamel, tubular dentine and predentine. Fibroblasts and lymphoid cells can be seen in the papilla. Arrow points to degenerated odontoblasts. Note the bone formation. Haematoxylin and eosin. ×415.

FIG. 10. Uncultured tooth bud 3 weeks after implantation beneath the oral mucosa. The formed matrix enamel and dentine are undergoing resorption. There is active bone formation in the papillary area. Haematoxylin and eosin. ×42.

FIG. 11. Higher magnification of part of Fig. 9 showing resorption of the matrix enamel (E) and dentine (D). Haematoxylin and eosin. ×415.

FIG. 12. Cultured tooth bud 1 week after implantation subcutaneously, showing matrix enamel, dentine and normal formative cells. There is no lymphoid infiltration. Haematoxylin and eosin. ×42.

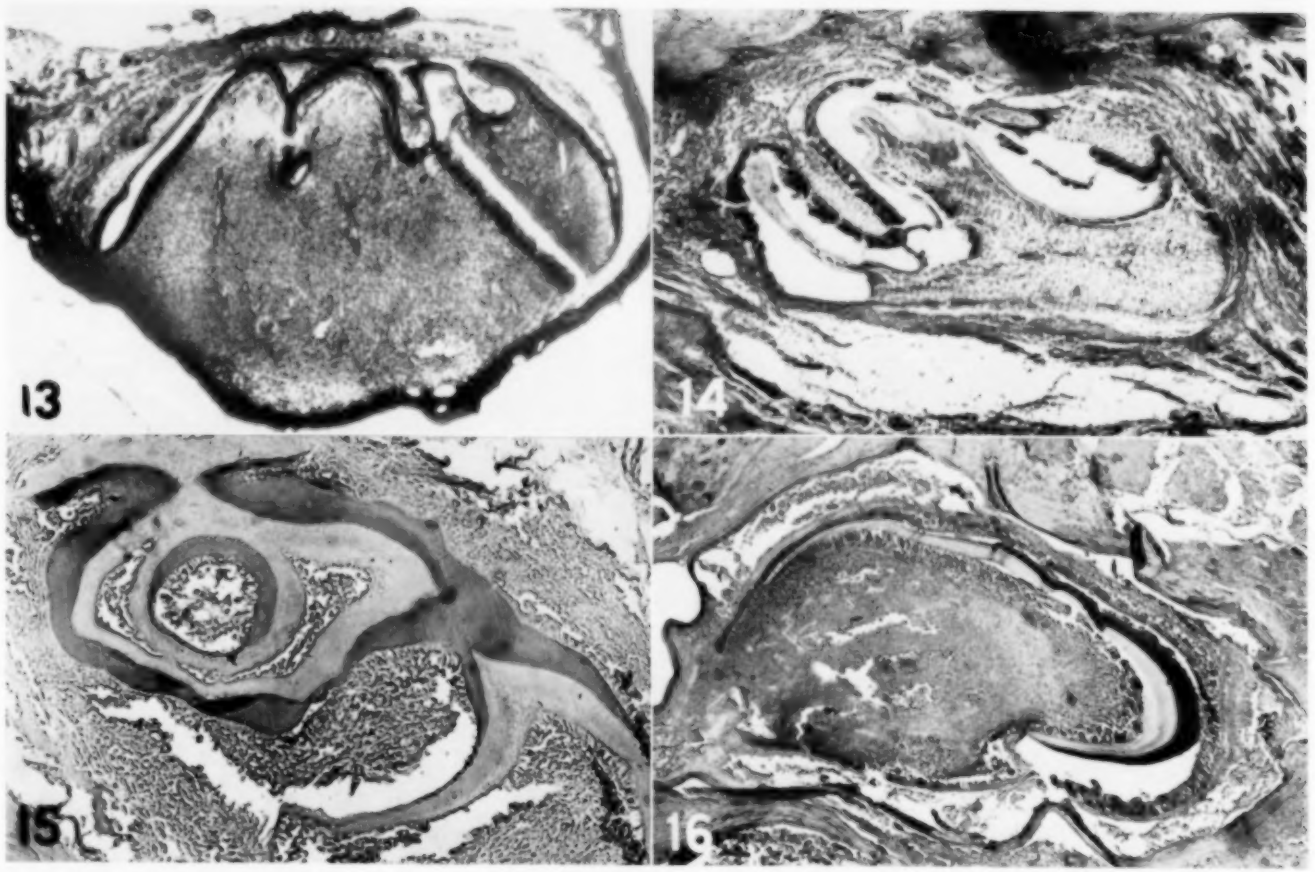

FIG. 13. Cultured tooth bud 2 weeks after implantation subcutaneously. Note morphological development and the formation of dentine and matrix enamel. The papilla is vascular, and there is no evidence of rejection. Haematoxylin and eosin. ×30.

FIG. 14. Cultured tooth bud 1 week after implantation beneath the oral mucosa. The morphologic development is normal, the papilla is vascularized, and there is no evidence of rejection. Haematoxylin and eosin. ×42.

FIG. 15. Cultured tooth bud 2 weeks after implantation beneath the oral mucosa. Section cut through crown. Morphology and development are normal. There is no evidence of rejection. Haematoxylin and eosin. ×42.

FIG. 16. Cultured tooth bud 6 weeks after implantation beneath the oral mucosa. The tooth lies in a bony crypt. The section is cut through a cusp and one root. All elements are normal. Note the bulbous growth centre of Hertwig's sheath. There is no evidence of rejection. The specimen was embedded in parlodion and sectioned at 20 mμ. The photomicrograph appears smudged because of the thickness of the section. Haematoxylin and eosin. ×21.

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ORGANIC MASS DISTRIBUTION IN BONE MATRIX UNDERGOING OSTEOCLASTIC RESORPTION

PRELIMINARY OBSERVATIONS

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Abstract—The technique of high-resolution ultrasoft microradiography has been applied to the study of the distribution of organic mass at sites of bone resorption. Preliminary observations on Versene-demineralized bone have revealed the presence of a band or rind of increased radio-density in the bone matrix at the point of junction between osteoclast and resorption front. This rind is approximately 1–1·5 μ in width, and in most preparations appears to have a striated structure.

A consideration of the technical methods employed leads to the conclusion that this structure does not coincide with the classically described striated border of KÖLLIKER. It is found only in association with osteoclasts which appear to be functionally active. Tentatively, it is suggested that this rind represents the site of accumulation of proteinaceous material, possibly reflecting the local concentration of those enzyme systems responsible for the dissolution of bone.

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THE INTRODUCTION of a method for attaining high-resolution X-ray images of histological sections at unit magnification (GREULICH and ENGSTRÖM, 1956; ENGSTRÖM *et al.*, 1957) has permitted the initiation of systematic microradiographic studies of organic mass distribution in a large number of soft tissues (ENGSTRÖM, LUNDBERG and BERGENDAHL, 1957; GREULICH, 1960). Recent application of this technique has provided new observations concerning organic mass distribution in bone matrix and its associated cellular constituents under a variety of conditions, both normal and pathological (GREULICH, 1959, 1960). The present report describes the results of preliminary microradiographic studies of organic mass distribution in areas of bone matrix undergoing osteoclastic resorption, and provides unique information regarding that structural area which is of the greatest importance in resorptive activities, namely the junction between the osteoclast and the bone.

MATERIALS AND METHODS

In the present studies, specimens of bone (knee joints and humeri) from rapidly growing mice and rats were fixed in 10% neutral formalin for 48 hr and were then demineralized in Versene (di-sodium EDTA, 34% aqueous solution, buffered with HCl to pH 7·4). Decalcification was carried out at 45°C for 4 days with constant mild agitation, and with a change of the chelating medium on alternate days. The samples were then washed in water, dehydrated through alcohols and were embedded in paraffin from xylol.

Sections for microradiography were cut at 6 μ and were mounted on Eastman Kodak Spectroscopic Plate No. 649–0, according to a technique described previously (GREULICH and ENGSTRÖM, 1956; GREULICH, 1960). X-ray exposures were carried out at from 1·25 to 1·75 kV, although the most satisfactory images were obtained at 1·5 kV after an exposure of 45 min (1·0 mA). The microradiographic apparatus employed a copper target and was adapted from that described by ENGSTRÖM and LUNDBERG (1957), as modified more recently in this laboratory (GREULICH, 1960).

Following exposure, the samples were removed by acetone, and the plate was developed, fixed and mounted under a cover glass for microscopic observation. Selected tissue samples were reclaimed from the acetone bath on an albuminized microscope slide, and were then stained by the Masson trichrome method for histological study. The illustrations which accompany this report provide the reader with a comparison between the histological tissue section and its companion microradiographic image.

RESULTS

Interpretation of microradiograms such as those shown in Figs. 2 and 4 is facilitated by recalling that, as in conventional radiograms, areas of greater radio-density appear under-exposed (white) in the developed plate, and in the positive prints used for the illustrations. For example, since the organic matrix of bone is constituted of a considerably larger concentration of the protein collagen than is the matrix of cartilage, the former is rendered white in the microradiogram, while cartilagenous zones tend to be darker in aspect. It is to be emphasized that the images seen in the microradiograms illustrated in Figs. 2 and 4 are those cast by organic chemical constituents of the tissues, the mineral material of bone and calcified cartilage having been removed by the process of chelation. Relative differences in contrast in the image, therefore, reveal comparable differences in the concentration of organic materials, usually of a protein character (in this case, primarily collagen), and their detection is made possible by the ultrasoft nature of the polychromatic X-ray spectrum which is utilized for registration of the image. At the same time, microscopical detail is provided both by the thinness of the histological section and, especially, by the high resolving power of the fine-grained Spectroscopic Plate. A full discussion of the theory and technique of ultrasoft microradiography may be found elsewhere (ENGSTRÖM, LUNDBERG and BERGENDAHL, 1957; ENGSTRÖM, 1959).

Figs. 1 and 2 compare respectively the stained histological section and the corresponding microradiogram of an area selected from the periphery of a mouse humerus. An immediate comparison may be made between the relative radio-densities of bone and cartilage matrices in the palisaded subepiphyseal spicules, as described above (Fig. 2). More important to the subject of this report, however, are the microradiographic features which are associated specifically with sites of osteoclasis. Examination of Fig. 1 reveals the presence of osteoclasts (arrows) eroding several of the subepiphyseal spicules.

Figs. 3 and 4 are higher power representations of the area enclosed by the box in Fig. 2, and demonstrate quite clearly both the histological and microradiographic

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appearance of an osteoclast in active bone resorption. The histological picture (Fig. 3) is typical of that seen at a site of bone resorption. The osteoclast lies partially within a well-demarcated Howship's lacuna which has presumably been formed by the bone lysing activity of the cell. Little detail is evident in the stained specimen with respect to the junctional region between cell and bone matrix, except for a slight increase of affinity for the green component of the trichrome stain, which appears as a somewhat darker linear zone in the illustration. The microradiogram (Fig. 4), on the other hand, demonstrates clearly the presence at this junction of a thin, somewhat striated band, approximately 1–1·5 μ in thickness, which exhibits an increased radio-opacity to the ultrasoft X-ray spectrum, and corresponds in position to the stained zone of Fig. 3. Close examination will reveal that this band or rind of increased radio-density is closely associated with the most superficial layer of bone matrix and has little, if any, connexion with the osteoclast cytoplasm.

These microradiographically demonstrable rinds have always been encountered at points in the bone against which an osteoclast is juxtaposed (Fig. 2, arrows). However, by comparing histological and microradiographic details, it has been possible to identify osteoclasts which are not associated with the rind. Such cells are for the most part, if not entirely, degenerating or inactive, and may be characterized by their relatively intense cytoplasmic acidophilia, and their possession of hyperchromic and occasionally pyknotic nuclei.

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DISCUSSION

Classical histological descriptions of the junctional zone between osteoclast and bone (KÖLLIKER, 1873) revealed the presence in this region of the so-called "striated border". This structure is currently regarded as representing a combination of the terminal portions of fine collagenous fibrils, which protrude from the surface of the resorbing bone, together with residual cytoplasmic processes of the osteoclasts (HANCOX, 1956). The significance of the collagenous portion has been held to lie in the fact that mineral and ground substance constituents of bone are relatively more labile to the lytic action of the osteoclast than is collagen (HAM, 1952). Therefore, fibrils of undegraded collagen may be encountered in the region of the osteoclast-bone matrix junction, and give rise to its striated appearance. With regard to the nature and origin of the cytoplasmic component, it has been suggested that in life there is a modification of the osteoclast plasma membrane where it adjoins the bone so as to form an undulating membrane (HANCOX, personal communication). Consequent to histological fixation, portions of the undulating membrane may remain firmly attached to the resorption front (despite the fact that the remainder of the cell commonly pulls away from it) and so contributes to the striated appearance of this region.

Interpretation of the microradiographic image described herein in terms of KÖLLIKER's striated border, i.e. collagenous fibrils and remnants of plasma membrane, is difficult to accept on several grounds. First, the rind of increased radio-density seems to be associated with the bone matrix proper rather than with the intermediate zone between osteoclast and bone. Second, although it is well established that the matrix of bone consists by weight almost entirely of collagen (EASTOE, 1956), the

microradiograms (Figs. 2 and 4) demonstrate that its radio-density is clearly less than that of the band or rind under discussion. For this reason particularly, it seems unlikely that the presence of the band can be ascribed to the excessive accumulation therein of collagen fibrils since, at most, these would be expected to be present in no greater concentration in the rind than they would be in bone matrix generally. The latter view is supported by the fact that electron micrographs do not suggest any local increase in the amount of collagenous material at the resorption front (SCOTT and PEASE, 1956). Lastly, the mass increment contributed by even a most complexly-folded plasma membrane would be insufficient to cause so marked an increase in radio-density.

Despite the fact that the rind does not appear to owe its absorptive capacity to collagen or plasma membrane, however, consideration of effects of the fixation and demineralization utilized in preparation of these specimens does lead to the tentative conclusion that the rind represents a focal accumulation of a proteinaceous material other than collagen.

Two possible explanations of this observation present themselves with reference to physiological significance. The first, and perhaps the most reasonable within the framework of current hypotheses favouring osteoclastic function by enzymatic action, is that the increased radio-density seen in the microradiograms is due, in part at least, to the presence of those enzymes concerned with bone destruction. Since there has as yet been no unequivocal demonstration of concentrated enzyme activity in the border of the resorption cavity (HANCOX, 1956; BURSTONE, 1960; MORSE and GREEP, 1960), such an explanation for these data must remain speculative.

A second possibility, of course, is that demineralization of this region is incomplete, and that the increased absorption of X-rays is due to the presence of calcium salts. In general, this seems unlikely, since the ultrasoft microradiographic method has proven to be a most sensitive method of testing for their presence. Some experience with microradiograms of partially decalcified bone suggests that even slight accumulations of mineral material show up as opaque, relatively coarse masses distributed more or less at random through both the bony and cartilagenous constituents of the section, while, in this case, the rind is a discrete structure specifically associated with osteoclasts. The combination of these facts appears to minimize the possibility that local increases in radio-density are due to inadequate demineralization.

On the other hand, current theories regarding both mineralization and demineralization have introduced the possibility that chelating mechanisms may be involved, at least in the initial stages of these processes (MCLEAN and URIST, 1955; GLIMCHER, 1960). It is well established that, just as certain divalent cations can displace others from chelate complexes, there is also a distinct difference in binding affinity for a given metal ion from one chelating compound to the next. Specimens utilized in the present study were demineralized by chelation with di-sodium EDTA which has only a moderate affinity for calcium (RUBIN, 1953). If a biological chelating mechanism (protein?) was active in osteoclastic resorption, and the chelate complex was retained after formalin fixation, it is possible that calcium would not be removed by the action of the exogenous histological demineralizing agent. In view of the high

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concentration of Versene employed for demineralization, however, this is a most speculative proposition, and needs much further study before it can be accepted or discarded.

The fact that these rinds are seen only in association with what are described classically as active osteoclasts, with large pale-staining nuclei and foamy, slightly granular cytoplasm, lends credence to the concept that they have functional significance. That they are not artifacts created by sectioning procedure is suggested both by their specific occurrence in relation to osteoclasts, as well as by the fact that they occur independently of the direction which the microtome knife took as it passed through the tissue block.

The intimate structural association between osteoclasts and areas of bone resorption has been recognized for over 100 years. Despite present-day acceptance that these cells are instrumental in osteolysis, the specific metabolic mechanisms involved in their activities remain undefined, principally because precise techniques for their study are unavailable. While the microradiographic observations presented above perhaps do little to elucidate the physiological mechanics of osteoclast function, they are offered because of their unique character, as well as in the hope that they will stimulate further interest in the nature of these yet enigmatic cells.

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FIG. 1. Masson trichrome-stained paraffin section of demineralized mouse humerus at border between periosteum and subepiphyseal spicules. ×380.

Bone matrix is more darkly-stained than cartilage. Osteoclasts are indicated by arrows.

FIG. 2. Microradiogram of section and field identical to that in Fig. 1. 1·5 kV, 1·0 mA, 45 min exposure. ×380.

Relative differences in radio-density of organic mass in matrices of cartilage and bone can readily be seen. Arrows indicate position of osteoclasts, as in Fig. 1. Note the thin bands or rinds of increased radio-density in bone matrix adjacent to these cells. The area outlined by the box is shown at higher magnification in Fig. 4.

FIGS. 3 and 4. Enlargements of histological section (Fig. 3) and corresponding microradiogram (Fig. 4), according to the outline indicated in Fig. 2. ×950.

Comparison of junctional zone between osteoclast and bone matrix reveals a slight increase in staining affinity at the edge of the matrix (Fig. 3), but a striking increase in organic radio-density in the corresponding microradiogram (Fig. 4, arrows). The striated structure of this rind of material is seen fairly clearly near the lower arrow.

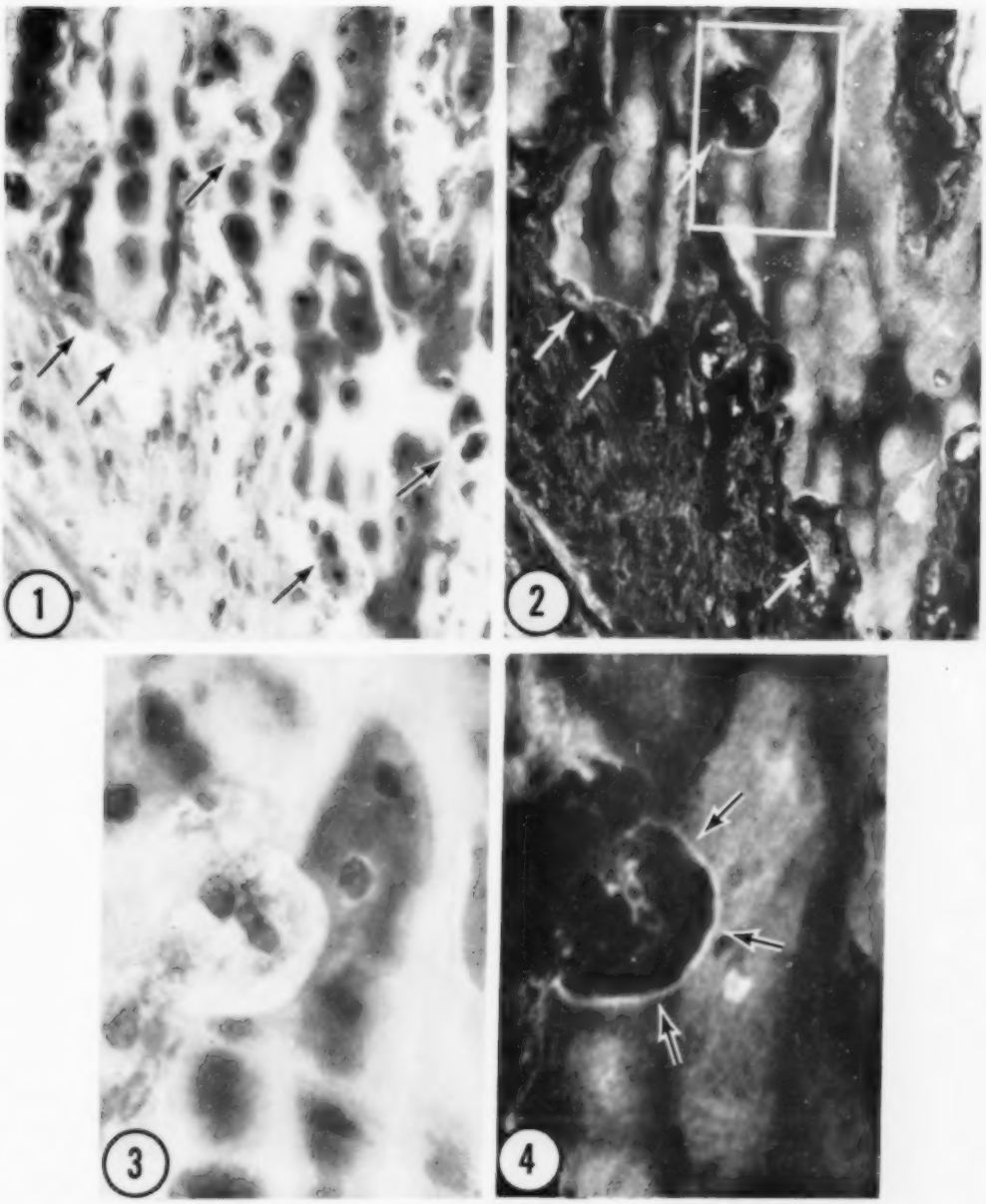

PLATE 1

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SHORT COMMUNICATION

EFFECT OF SELENIUM ON DENTAL CARIES IN THE RAT

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FROM THE RESULTS of a number of epidemiological studies on dental caries conducted in the State of Oregon among school children, it was shown for the first time that the trace element selenium may be a factor responsible in bringing about an increased susceptibility to dental caries attack (HADJIMARKOS, STORVICK and REMMERT, 1952; HADJIMARKOS and BONHORST, 1958). Recently, an investigation on the selenium–caries relationship was conducted in the State of Wyoming which contains extensive areas of seleniferous soils. The findings demonstrated that children born and reared in seleniferous areas had higher caries rates in comparison with subjects residing in non-seleniferous areas of the State (TANK and STORVICK, 1960).

In a recent report, MUHLER and SHAFER (1960) showed that the addition to a stock corn cariogenic diet of 20 per cent irradiated yeast at the expense of corn, when fed to weanling rats for a period of 100 days increased significantly the incidence of caries and, as well, reduced body growth in comparison with the controls. In a previous experiment (BUTTNER and MUHLER, 1959), it was found that when yeast was ashed and then added to the diet both effects, cariogenic and growth-inhibiting, were lost. As a result of this, it was suggested that a heat-labile factor present in yeast was responsible for the observed effects.

In some previous investigations, it was shown that selenium was the active component of Factor 3 in dried brewers yeast which prevents exudative diathesis in chicks and dietary liver necrosis in rats (SCHWARZ *et al.*, 1957; SCHWARZ and FOLTZ, 1957). However, when ashed yeast was added to the diet of chicks it failed to provide protection against exudative diathesis. This decrease in activity of ashed yeast suggested that during the ashing process selenium was either lost to a significant extent or that it was converted into a less active form (REID *et al.*, 1958; KELLEHER *et al.*, 1959). In view of the fact that yeast is an important source of selenium and that ashing reduces its activity, it was thought advisable to determine the selenium content of the corn-cariogenic diet, irradiated yeast and ashed yeast for an explanation of the findings reported by MUHLER and SHAFER (1960).

The irradiated yeast was preheated in an oven at 130°C for 24 hr and then ashed in a muffle furnace at 550°C for 24 hr. Selenium determinations in samples of the above materials were made by the neutron activation analysis method at the Oak

Ridge National Laboratory. The selenium content of the stock corn diet was found to be 0·13 p.p.m., of the irradiated yeast 1·16 p.p.m. and of ashed yeast 0·74 p.p.m. The selenium in the stock diet is apparently contributed mainly by corn since it is known that grains produced in seleniferous areas of the country accumulate considerable amounts of this element (TRELEASE and BEATH, 1949, p. 221). On the basis of the analyses, it is estimated that the selenium content of the corn diet including 20 per cent yeast would be about 0·34 p.p.m. This amount of selenium present in the diet of the rats may seem small. It should be pointed out, however, that the intake of minute amounts of selenium exerts profound effects. It has been shown that the maximum growth in chicks fed a vitamin E-free Torula yeast diet was obtained by adding selenium to the basal diet, as sodium selenate, to a level of 0·05 p.p.m. On the other hand, when the diet contained selenium at a level as low as 0·1 p.p.m. it resulted in some retardation of growth (RAHMAN *et al.*, 1960). Increasing the level of selenium as selenate to 1·0 p.p.m. in the basal diet of the chicks not only produced pronounced depression in growth but evidence of toxicity was also observed (REID *et al.*, 1958).

It thus appears that in the experiments of MUHLER and SHAFER (1960) and BUTTNER and MUHLER (1959) the increase in the level of selenium contributed by the addition of considerable amounts of yeast to the stock corn diet may be responsible for bringing about both the increase in the incidence of caries and growth depression which they observed in their animals. This is particularly so since these workers used weanling rats and it has been observed that young rats are much more susceptible to selenium intake than older ones (TRELEASE and BEATH, 1949, p. 165).

Although the possible dangers to health from the cumulative effects of daily ingestion of small amounts of selenium have been recognized (TRELEASE and BEATH, 1949, p. 221), our knowledge about the selenium intake that may be tolerated without ill effects is still very meagre. The findings of the epidemiological studies on the selenium–caries relationship, mentioned above, indicate that further research is needed in this area.

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THE MICROSTRUCTURE OF YOUNG DENTINE

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Abstract—Photomicrographs of a 20 μ thick ground section of young undecalcified dentine show peritubular relative refringency effects known as "Becke" effect. Historadiographs of this young dentine show peritubular radiopaque rings indicative of peritubular calcification of young coronal dentine.

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INTRODUCTION

SALTER (1865) described the appearance of dentinal tubules seen in ground sections as follows: "The dentinal tubules have extremely thin walls, which can be seen in profile, but which, in transverse section, as viewed by high powers of the microscope, display a broad brilliant ring of considerable thickness around each. This appearance is an optical illusion and has given rise to much error in interpreting the histological elements of dentine." SALTER further states that HENLE in 1841 described the brilliant ring around dentinal tubules merely as an optical illusion.

MILLER (1954), BRADFORD (1955) and BLAKE (1958) showed bright rings around the dentinal tubules in their photomicrographs of ground dentine sections. These bright rings were assumed to be structures instead of optical effects. They also showed historadiographs with bright or radiopaque rings about the radiolucent dark shadows of the dentinal tubules. They claimed that these peritubular radiopaque rings represented increased peritubular calcification. BERGMAN and ENGFELDT (1955) also used light photomicrographs and historadiographs to show cross-sections of dentine. The ground sections they showed were made from dentine taken between an area of caries and the pulp. They were of the opinion that the bright rings about the dentinal tubules shown in both the photomicrographs and historadiographs represented peritubular calcification. They attributed these peritubular effects to dentine sclerosis stimulated by the adjacent dental caries. ROCKERT (1956), on the other hand, using a similar historadiographic method also showed radiopaque peritubular rings which he claimed were peritubular calcifications and attributed them to age changes. FRANK (1959), using the electron microscope and undecalcified sections of dentine from an erupted young adult human third molar, showed areas of peritubular calcification. NALBANDIAN, GONZALES and SOGNNAES (1960) showed that dentine tubules in ground sections from the roots of teeth are surrounded by light translucent radiopaque rings.

This brief review of the literature indicates that there is a difference of opinion about the microstructure of dentine when photomicrographs and historadiographs of ground sections are examined. Bright peritubular rings seen in photomicrographs of ground sections of dentine are assumed to represent both increased

calcification and optical illusory effects. The presence of uniform peritubular radiopaque rings about the dentinal tubules in historadiographs are indicative of increased calcification. This increased peritubular calcification has been interpreted as a hypercalcification attributed both to a reaction to age and to a pathologic lesion such as dental caries.

It is the purpose of this study to examine the coronal dentine of a newly formed unerupted permanent tooth with light micrographic and X-ray micrographic methods.

METHOD AND MATERIAL

An unerupted human premolar tooth removed for orthodontic reasons from a 10 year old female was held with a machinist's clamp, mounted on a jeweller's lathe, and the coronal surfaces ground flat with a mounted wet carborundum wheel. The axial coronal surfaces were ground flat and perpendicular to the ground flat occlusal surface. As a result of this grinding procedure all of the enamel was removed from all coronal surfaces of the tooth. A thin steel circular saw was then used to slice several $\frac{1}{2}$ mm thick cross-sections along a line parallel to the ground occlusal surface of the tooth. These relatively thick cross-sections were cut through the crown parallel to and between the flattened occlusal surface and the pulp chamber proper. Each section was then reduced to a thickness of 20 μ and made plano-parallel. This reduction was accomplished with finger pressure and a levigated alumina slurry on a glass plate. Final grinding and polishing was accomplished with an Arkansas stone, with water as the wetting agent.

The ground dentine section used in this study was from one of the deeper occlusal plane slices, that is one cut through or across the labial and palatal pulp horns of the young tooth. This 20 μ thick section was then cut in half in a bucco-palatal direction through the centres of the pulp horn openings (Fig. 1a). The section was thus halved for convenience in handling while making the low voltage contact radiograph, the first step in the preparation of historadiographs.

The contact radiograph of the dentine ground section was made with a low voltage X-ray generator (Phillips Microradiographic Unit), using a fine grained Kodak spectrographic film type 649–0. Successively higher magnifications of the contact radiograph (historadiographs) are seen in Figs. 1 (a), (b) and (c).

Following the making of the contact radiograph the ground section was mounted on a glass slide with Permount and covered with a coverslip. Two photomicrographs of the section were made using a 4 mm objective, a 5x eyepiece and an Orthophot photomicrographic camera (Fig. 2). The first exposure was made with the microscope focused on the upper surface of the ground section and is illustrated in Fig. 2 (a). Then the tube of the microscope was lowered with the fine focusing mechanism and with the section still in focus another exposure was made and is illustrated in Fig. 2 (b). The two photomicrographs illustrated were taken in an area of about 100 μ diameter in the centre of the ground section between the pulp horns. The plane of the section was parallel to the occlusal surface and perpendicular to the dentinal tubules which extend from between the base of the pulp horns to the occlusal dentino–enamel junction. The original magnification was about 400 diameters.

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OBSERVATIONS

A. *Historadiographs*

Successively higher magnifications of the low voltage contact radiograph of the ground dentine section are illustrated in Figs. 1 (a), (b) and (c). The first historadiograph represents about a 50× magnification of the contact radiograph and is illustrated in Fig. 1 (a). One can see the X-ray shadows of the halved buccal and palatal pulp horns of the unerupted premolar tooth. There is a relatively radiolucent 100 μ wide band of dentine about the borders of both pulp horn margins. The cross-cut dentinal tubules are not resolved at the magnification of the dentine section contact radiograph shown in Fig. 1 (a).

The contact radiograph is again shown at 100× magnification in Fig. 1 (b). This historadiograph shows only the palatal pulp horn shadow and some of the surrounding cross-cut dentinal tubules. One can just see that the area of relative radiolucency of the dentine about the pulp horn shadow margin is due to the fact that the dentinal tubules appear to be larger than they are farther out in the dentine.

The historadiograph of the ground section of dentine, at 250× magnification, is shown in Fig. 1 (c). This illustration does not show the shadow of one of the pulp horns which would be above and slightly to the right. In the upper half of this illustration dark radiolucent dentine tubule lumina can be seen. The tubules in the upper half of the picture are surrounded by the X-ray opaque, grey, calcified dentine matrix. The lower half of the illustration shows what appears to be smaller dentinal tubule lumina surrounded by narrow peritubular rings of X-ray opacity. These X-ray opaque peritubular rings signify peritubular hypercalcification.

Since the dentine closest to the pulp chamber represents dentine most recently formed, this historadiograph indicates that the rings of peritubular calcification appear after the matrix of the dentine becomes initially calcified. These historadiographs of this young clinically unaffected dentine also demonstrate the fact that peritubular calcification of the major portion of the dentine of a young tooth is quite normal.

B. *Photomicrographs*

That thin ground sections of dentine can be made to show what appear to be differences in structure by slight differences in focus of the microscope is illustrated in Fig. 2. The optical illusion produced in this manner, although not generally recognized, has been pointed out before (SALTER, 1865). The bright area about the dark tubule centres in Fig. 2 (a) and the bright tubule centre with the dark border seen in Fig. 2 (b) represent two different manifestations of the "Becke" effect (OSTER and POLLISTER, 1955). The "Becke" effect is brought about by a relative refringency of ordinary light rays striking a boundary perpendicular to the slide (dentinal tubule wall) between two media of different indices of refraction (mounting media and dentine).

The "Becke" effect and the secondary tubule curvature make it difficult, using undecalcified histologic sections, to determine the true microstructure of dentine. Unless the sections are cut perpendicular to the dentinal tubule axis and thin enough,

that is within the length of the secondary curvature and focused sharply to rule out the "Becke" effect, one cannot get a true picture of the tubule size and margins.

If the microscope is focused high on the ground section, the tubules will appear to have dark centres. These dark centres should not be interpreted as Tomes fibres.

Since the dentine used in this study was taken from an unerupted premolar tooth of a 10 year old female, it was not affected by oral stimuli such as abrasion, attrition or caries. The peritubular calcification is normal throughout the greater portion of the dentine and cannot be attributed to a reaction to injury, such as those which accompany abrasion, attrition or dental caries, or to an age change.

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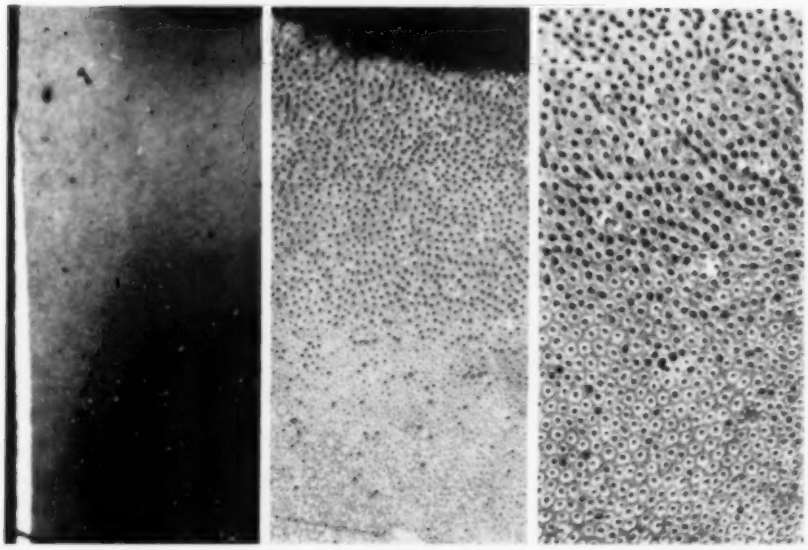
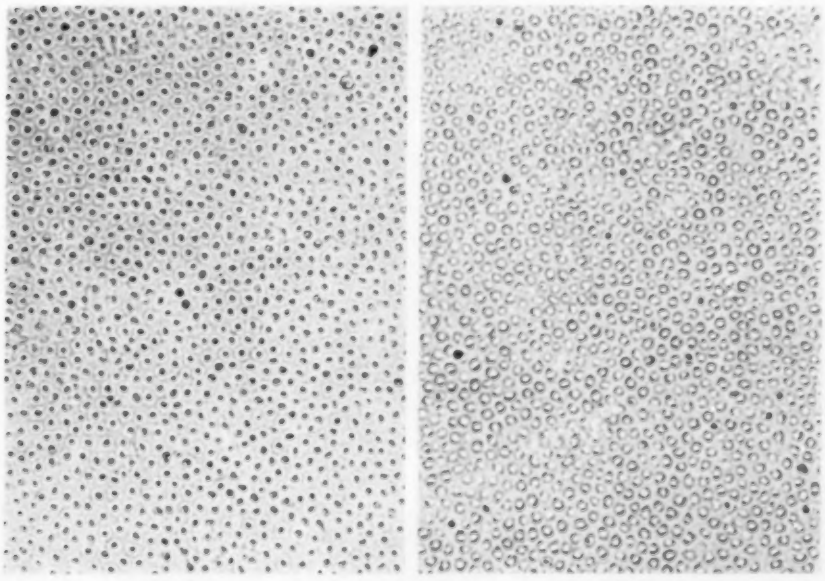


(a) (b) (c)

FIG. 1. (a) A historadiograph of the ground section cut through the palatal (above) and buccal (below) pulp horns. ×50. (b) Further magnification of (a) with the shadow of palatal pulp horn above. ×100. (c) High magnification of the contact radiograph. ×250.



(a) (b)

FIG. 2. Photomicrographs of the ground section of dentine taken (a) with the objective high and (b) with the objective lowered. ×300.

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THE FREE AMINO ACID COMPOSITION OF HUMAN SALIVA

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Abstract—The quantitative free amino acid composition of whole saliva and parotid and submaxillary secretions collected on 3 or 4 separate days was determined in three individuals. Free amino acids were determined semiquantitatively in the whole saliva of six individuals collected on 10 separate days over a period of 3 weeks. Eighteen free amino acids were found in whole saliva, with the concentration of some amino acids varying considerably in individual salivas. Six amino acids were found consistently and three inconsistently and in very small quantities in parotid secretions. Twelve amino acids were found regularly and two irregularly in submaxillary secretions. A comparison of whole saliva and submaxillary secretion from an individual indicates that approximately one-half of the free amino acids in whole saliva are submaxillary in origin. The ammonia nitrogen, amino nitrogen and total nitrogen were determined in the whole saliva of six individuals.

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INTRODUCTION

AS INDICATED by a number of investigations, free amino acids are present in whole human saliva. KESEL *et al.* (1947), utilizing biological assay methods, found eleven amino acids in autoclaved, whole saliva. KIRCH *et al.* (1947), extending the previous investigation, found sixteen amino acids in autoclaved, whole human saliva. No correlation was found in either investigation between salivary amino acid content or concentration and caries susceptibility. Whereas the salivary amino acids decreased, nevertheless they were always present in measurable quantities, even when individuals were on a low protein diet (KIRCH *et al.*, 1950).

There has been much investigation of a possible correlation between the presence or absence of tryptophan in saliva and caries susceptibility. KIRCH *et al.* (1947), utilizing a *Lactobacillus* species for the determination of free tryptophan, found no relationship between salivary tryptophan and caries activity. TURNER and CROWELL (1947), on the other hand, utilizing a glyoxylic–sulphuric acid method for the detection of both free and bound tryptophan (SHAW and MACFARLANE, 1938, 1940), concluded that there was an inverse relationship between the presence of a salivary tryptophan-like substance (or similar indole-containing molecules), the rate of breakdown of starch and caries susceptibility. When STACK (1954) determined both free and bound salivary tryptophan with *p*-dimethyl-amino-benzaldehyde in a strongly acid solution, he often found a direct relationship between tryptophan concentration and caries incidence, in contradiction to TURNER and CROWELL (1947). When EDMONDS, BELL and BEERSTECHER (1957) utilized *Leuconostoc mesenteroides* as the biological indicators for free tryptophan, they found less in saliva than had been previously reported and detected no relationship between caries incidence and salivary tryptophan levels.

Applying chromatographic techniques to ultrafiltrates of fresh, whole human saliva, GOLDBERG, GILDA and TISHKOFF (1948) found thirteen free amino acids. ROSEBEEK (1949), also utilizing chromatographic techniques, detected fourteen amino acids in the ultrafiltrates of whole human saliva. Apparently unaware of some of the previous investigations, MOOR and GILLIGAN (1951), by chromatographic analysis, consistently found only four amino acids in alcohol filtrates and dialysates of whole, stimulated human saliva and occasionally detected a few other amino acids. Although peptides were not specifically identified by their chromatographic procedures, hydrolysis of salivary components, presumably peptides, caused as much as an eight-fold increase in the amino acids. WOLDRING (1955) found eighteen free amino acids by analysing ultrafiltrates of whole, stimulated human saliva by quantitative chromatographic techniques. WIEL (1957), utilizing qualitative chromatographic methods on alcohol deproteinized secretions of human parotid and submaxillary glands, found ten amino acids constantly present in each secretion and fourteen amino acids in submaxillary secretions. The amino acid content of parotid secretion was much less than that of submaxillary secretion. Similar secretions from different individuals did not differ appreciably as to amino acid content and no difference was noted between the amino acid content of the saliva of men and women. ROSE and KERR (1958), using semiquantitative paper chromatography, determined the amino acids of whole saliva and parotid and submaxillary secretions of normal individuals and the parotid secretion of individuals with metabolic bone disease, cystinuria and polycythaemia vera. Little difference was found between the parotid secretion of normal and diseased individuals. Ten amino acids were identified in whole saliva, eight in parotid and six in submaxillary secretions. Considerable phosphoethanolamine was found in parotid and submaxillary secretions and less was present in whole saliva.

Previous investigations have not been in very close agreement as to the amino acid composition of human saliva. Because whole saliva, which had circulated for an indefinite period of time in the oral cavity, was analysed in most investigations, such analyses would not differentiate between normally secreted amino acids and those derived from the breakdown of salivary protein or peptides after saliva had entered the oral cavity. In addition, the composition of salivary secretions may vary in an individual or between individuals. In extending previous investigations, repeated quantitative and semiquantitative determinations of eighteen amino acids by chromatographic techniques were made of whole saliva and of the secretions of parotid and submaxillary glands of three individuals. Determinations were also made of the ammonia, amino and total nitrogen of whole salivas of six individuals.

METHODS

For the semiquantitative and quantitative determination of free amino acids, whole saliva and salivary gland secretions were obtained from randomly selected volunteers immediately after brushing the teeth and approximately 2 hr after breakfast. The samples collected were whole saliva and parotid secretion obtained with paraffin stimulation and submaxillary secretion obtained with stimulation of the oral mucosa by 2% acetic acid. Paraffin-stimulated parotid secretion was collected by

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means of a Curby cup (CURBY, 1953). Submaxillary secretion was collected by means of an elliptical-shaped cup (Fig. 1), made of copper and lined with hard wax to conform with the oral mucosa surrounding the orifices of the ducts. During the period of collection, submaxillary secretion was obtained while swabbing the tongue with 2% acetic acid. By this procedure 10–15 ml of submaxillary secretion could be obtained in approximately 20 min.

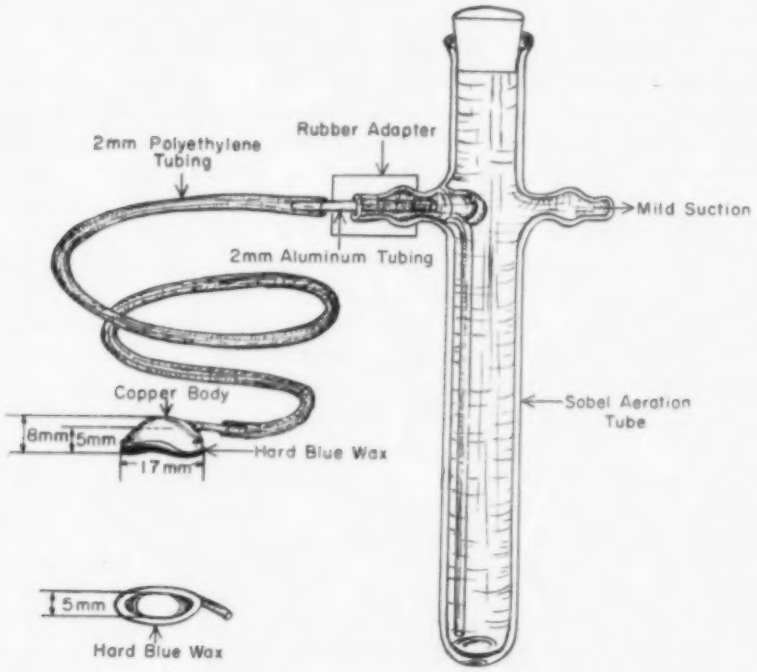

FIG. 1. Apparatus for obtaining submaxillary secretion.

The caries susceptibility status of the individuals studied was determined by the salivary lactobacillus counts (ROGOSA, MITCHELL and WISEMAN, 1951) and by Snyder's test (SNYDER, 1940).

For the analysis of whole saliva, 30 ml were collected with paraffin stimulation in sterile tubes immersed in ice. For chromatographic procedures, ultrafiltration was begun immediately in Visking dialysing cylinders at 1–2°C under nitrogen at a pressure of 200 mm of mercury. After 24–30 hr, approximately two-thirds of the saliva passed through the membrane as a clear, colourless fluid. During the period of filtration, the ammonia and amino nitrogen content of the saliva remained constant. After filtration was terminated, the filtrate was dried *in vacuo* in a watch glass over sulphuric acid at 5°C for 48–72 hr. The dried residue was then dissolved in a minimal amount of distilled water. 30% hydrogen peroxide was added to equal one-fifth of the

final volume which never exceeded 2 ml. Oxidation of the sample and chromatographic analysis were then performed as previously described by BATTISTONE and BURNETT (1956). In the previous study tyrosine values were lowered by oxidation but they were not significantly diminished in the present study and were reproducible. For determination of the amino acid composition of each saliva sample, three 300–500 μl portions of the oxidized residue were each analysed separately by two dimensional chromatography, along with three separate analyses of the composite solution of eighteen amino acids for reference standards. The chromatographic procedures were performed at a temperature of 20°C and relative humidity of 42±3 per cent. In the semiquantitative determination of amino acids, visual comparisons and estimations were made of the colours which developed on the chromatograms; for quantitative determinations, the colour of each spot was eluted and its intensity was determined spectrophotometrically. Standard concentrations of the eighteen amino acids analysed ranged from 2·0 to 7·6 mg/20 μl. The variation inherent in the quantitative analysis of these concentrations resulted in coefficients of variation ranging from 4·3 to 14·3 per cent, based on twelve determinations of each amino acid. The average coefficient of variation for the eighteen amino acids was 9·7 per cent. Total nitrogen and ammonia nitrogen were determined by the methods of SOBEL, MAYER and GOTTFRIED (1944) and amino nitrogen was determined by the method of SOBEL, HIRCHMAN and BESMAN (1945).

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RESULTS

In order to establish the relative frequency of occurrence in saliva of the eighteen free amino acids subsequently analysed for on a quantitative basis, a preliminary semiquantitative analysis was made of these acids in the whole saliva of six individuals collected on 10 separate days, extending over a period of 3 weeks (Table 1). The individuals were picked at random and their caries status was not considered at the time of the analyses. All were males, ranging in age from 19 to 42 years of age. At least five saliva samples from each individual were analysed separately on one dimensional chromatograms in order to separate and identify phenylalanine, leucine and isoleucine, which occurred together in the two-dimensional chromatograms. The amino acids appearing consistently were aspartic, glutamic, threonine, serine, glycine, alanine, phenylalanine, leucine and isoleucine. There was considerable inconsistency in proline, cystine, valine, methionine, tyrosine, tryptophan, histidine, lysine and arginine both in saliva of an individual from day to day and between the salivas of different individuals on the same or different days.

The salivas of three individuals were used in the next series of analyses. One of these individuals (G.C.B.) was a male, 31 years of age, who was caries insusceptible in that his lactobacillus counts were consistently less than 100 per ml of saliva and he had an indefinitely negative Snyder test. Another individual (J.A.Z.) was a male, 40 years of age, who was moderately caries susceptible in that his lactobacillus counts ranged from 10,000 to 20,000 per ml of saliva and his Snyder tests were positive within 48 hr but not 24 hr. The other individual (G.K.) was a male, 30 years of age, whose lactobacillus counts consistently exceeded 60,000 per ml of saliva and whose

TABLE 1. THE OCCURRENCE OF FREE AMINO ACIDS IN TEN PORTIONS OF WHOLE SALIVA COLLECTED FROM EACH OF SIX INDIVIDUALS ON 10 SEPARATE DAYS

Amino acids	G.C.B.	J.A.Z.	J.E.	G.H.	G.K.	G.W.B.
Aspartic	10*G*	10*G*	10*G*	10*G*	10*S*	10*G*
Glutamic	10*S*	9*G*	10*S*	10*G*	10*S*	10*G*
Threonine	10*S*	10*S*	10*S*	10*S*	10*S*	10*S*
Serine	10*G*	10*S*	10*S*	10*S*	10*G*	10*S*
Proline	5*W*	0	0	3*W*	1*W*	3*W*
Glycine	10*S*	10*S*	10*S*	10*S*	10*S*	10*G*
Alanine	10*S*	10*S*	10*S*	10*S*	10*S*	10*S*
Cystine	0	4*W*	0	0	0	1*W*
Valine	8*F*	9*S*	6*F*	8*G*	4*W*	10*G*
Methionine	0	3*W*	2*W*	0	0	0
PLI*	10*S*	10*S*	10*S*	10*S*	10*S*	10*S*
Tyrosine	3*W*	6*W*	0	5*W*	3*W*	0
Tryptophan	0	0	2*W*	1*W*	3*W*	2*W*
Histidine	0	3*W*	3*W*	0	1*W*	0
Lysine	9*G*	7*W*	8*F*	5*W*	2*W*	5*F*
Arginine	9*F*	6*W*	8*F*	3*W*	7*F*	4*F*

* Phenylalanine, leucine and isoleucine.

Numerals represent the number of times each amino acid was detected. Letters indicate the relative concentration of the amino acid as established by visual comparisons with standards (*S*= strong; *G*=good; *F*=fair; *W*=weak).

Snyder tests were positive within 24 hr. Quantitative values for the free amino acids in whole saliva and submaxillary secretions are shown in Tables 2 and 3. Whole saliva from each individual was analysed on 4 separate days (Table 2). The range of the values obtained on separate days is considerable, varying in some instances between relatively large amounts and complete absence. In some salivas traces of cystine, histidine, tryptophan and methionine were detected occasionally but not consistently. Histidine was found regularly in the saliva of only one individual. The data in the respective tables were analysed statistically for significance. When critical ratios (difference between the means divided by the standard error) were calculated for comparison of the amino acid composition of the three salivas, few significant differences were found and they formed no consistent pattern in regard to caries susceptibility or immunity.

The concentrations of free amino acids found in submaxillary secretions are shown in Table 3. The submaxillary secretions analysed were collected on 3 alternate days from the three individuals. The twelve amino acids found consistently were aspartic, glutamic, glycine, serine, threonine, alanine, arginine, lysine, valine, phenylalanine, leucine and isoleucine. Methionine and proline were found occasionally, sometimes in only trace amounts. Variations in concentration of amino acids in submaxillary

secretions were often as great and, in many instances, greater than they were in the whole saliva of the same individual. Critical ratios calculated for comparison of the amino acid composition of submaxillary secretions indicate some random significant differences but such differences were not consistent and formed no definite pattern.

TABLE 2. THE FREE AMINO ACID CONTENT OF WHOLE STIMULATED SALIVA COLLECTED ON 4 SEPARATE DAYS FROM THREE INDIVIDUALS (VALUES EXPRESSED AS MILLIGRAMS PER CENT)

Amino acids	G.C.B.		J.A.Z.		G.K.	
	Mean and standard error	Coefficient of variation	Mean and standard error	Coefficient of variation	Mean and standard error	Coefficient of variation
Cystine	0·00		(trace)		0·00	
Aspartic	0·21 ± 0·054	47·6	0·18 ± 0·043	44·4	0·09 ± 0·052	111·1
Glutamic	0·38 ± 0·085	47·4	0·32 ± 0·122	75·0	0·47 ± 0·135	59·6
Glycine	0·83 ± 0·203	48·2	1·02 ± 0·115	21·1	0·83 ± 0·184	45·8
Serine	0·17 ± 0·061	70·6	0·12 ± 0·060	100·0	0·34 ± 0·045	23·5
Threonine	0·28 ± 0·044	28·6	0·19 ± 0·026	21·1	0·39 ± 0·013	5·1
Alanine	0·28 ± 0·063	42·8	0·34 ± 0·088	52·9	0·37 ± 0·022	10·8
Arginine	0·14 ± 0·036	57·1	0·21 ± 0·072	85·7	0·23 ± 0·081	69·6
Lysine	0·12 ± 0·046	83·3	0·51 ± 0·185	82·4	0·18 ± 0·086	100·0
Histidine	0·00		0·29 ± 0·101	69·0	0·00	
Tryptophan	(trace)		0·01*		0·00	
Valine	0·15 ± 0·064	80·0	0·17 ± 0·077	117·6	0·22 ± 0·016	45·4
PLI	0·77 ± 0·076	20·8	1·03 ± 0·070	13·6	0·74 ± 0·066	18·9
Tyrosine	0·05 ± 0·025	80·0	0·38 ± 0·095	47·4	0·19 ± 0·054	52·6
Proline	0·06 ± 0·050	166·7	0·00		0·00	
Methionine	0·00		0·01*		0·00	
Total amino acids	3·44 ± 0·402	23·4	4·78 ± 0·310	13·1	4·06 ± 0·230	11·3

* Found in only one in four saliva samples.

A comparison was made of the free amino acid composition of whole saliva and submaxillary secretion collected within a 1 hr period from the same individual (G.C.B.) used in previous studies. These data (Table 4) indicate that about one-half of the free amino acids in saliva are accounted for in the submaxillary secretion. Also analysis was made of the free amino acid composition of the parotid secretion of three individuals (Table 5). Many of the amino acids detected in whole saliva and submaxillary secretion could not be found in parotid secretion. Glutamic acid, glycine, alanine, phenylalanine, leucine and isoleucine were detected consistently in the parotid secretions obtained from each of the three individuals. Aspartic acid, valine and serine were found occasionally in parotid secretions.

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TABLE 3. THE FREE AMINO ACID CONTENT OF SUBMAXILLARY SECRETIONS COLLECTED ON 3 SEPARATE DAYS FROM THREE INDIVIDUALS (VALUES EXPRESSED AS MILLIGRAMS PER CENT)

Amino acids	G.C.B.		J.A.Z.		G.K.	
	Mean and standard error	Coefficient of variation	Mean and standard error	Coefficient of variation	Mean and standard error	Coefficient of variation
Aspartic	0·16 ± 0·030	33·1	0·24 ± 0·047	34·1	0·09 ± 0·029	55·5
Glutamic	0·12 ± 0·038	55·8	0·08 ± 0·014	31·2	0·14 ± 0·018	22·8
Glycine	0·17 ± 0·018	18·8	0·34 ± 0·071	36·1	0·40 ± 0·023	10·0
Serine	0·15 ± 0·098	114·0	0·18 ± 0·081	78·3	0·31 ± 0·042	23·8
Threonine	0·05 ± 0·032	112·0	0·12 ± 0·040	58·3	0·13 ± 0·017	22·3
Alanine	0·08 ± 0·042	97·5	0·08 ± 0·026	56·2	0·27 ± 0·015	9·6
Arginine	0·01 ± 0·013	230·0	0·02 ± 0·010	85·0	0·00	
Lysine	0·03 ± 0·017	100·0	0·02 ± 0·009	80·0	0·22 ± 0·033	25·9
Valine	0·05 ± 0·014	50·0	0·10 ± 0·023	40·0	0·16 ± 0·023	25·0
PLI	0·25 ± 0·040	28·0	0·23 ± 0·063	47·8	0·27 ± 0·131	84·0
Methionine	0·00		(trace)			
Proline	0·00		0·04 ± 0·04	175·0	0·00	
Total amino acids	1·09 ± 0·064	10·1	1·44 ± 0·070	8·4	1·98 ± 0·170	14·7

TABLE 4. THE FREE AMINO ACID CONTENT OF WHOLE AND SUBMAXILLARY SALIVA COLLECTED WITHIN 1 hr FROM THE SAME INDIVIDUAL (G.C.B.) (EXPRESSED AS MILLIGRAMS PER CENT)

Amino acids	Whole saliva	Submaxillary saliva
Cystine	Not detected	Not detected
Aspartic	0·06	0·08
Glutamic	0·39	0·17
Glycine	0·98	0·36
Serine	0·05	0·14
Threonine	0·17	0·05
Alanine	0·40	0·04
Arginine	0·05	Not detected
Lysine	0·06	Not detected
Histidine	Not detected	Not detected
Tryptophan	Not detected	Not detected
Valine	0·23	0·11
PLI	0·71	0·53
Tyrosine	0·14	0·07
Proline	Not detected	Not detected
Methionine	Not detected	Not detected
Total	3·24	1·55

TABLE 5. FREE AMINO ACID CONTENT OF PAROTID SALIVA FROM THREE INDIVIDUALS (EXPRESSED AS MILLIGRAMS PER CENT)

Amino acids	G.C.B.	J.A.Z.	G.K.
Aspartic	—	—	0·01
Glutamic	0·01	0·01	0·02
Serine	—	—	Uncertain
Glycine	0·02	0·01	0·01
Alanine	0·01	0·01	0·01
Valine	Uncertain	0·01	Uncertain
PLI	0·02	0·02	0·02

A study was made also of the ammonia, amino and total nitrogen of six individuals (Table 6). Three of these individuals (Z.A., B.E., C.H.) were rated as caries susceptible by standards used for the individuals in the first quantitative analysis of whole saliva. The other three individuals (C.D.Y., E.C., F.C.) were caries insusceptible. The ammonia nitrogen content of the salivas ranged from a maximum of 11·4±0·84 mg per cent to a minimum of 4·0 mg per cent. Amino nitrogen ranged from 3·4±0·23 to 1·5±0·16 mg per cent. The highest nitrogen content was 93·4±6·6 mg per cent, while the lowest was 49·9±3·4 mg per cent. Statistical evaluation of the data indicates that the individual values for ammonia nitrogen were significantly different in twelve of fifteen possible comparisons. The values for the individual amino nitrogens were significantly different in eight of fifteen possible comparisons. When individual values for total nitrogen were compared, they varied significantly in ten of fifteen possible comparisons.

TABLE 6. AMMONIA, AMINO AND TOTAL NITROGEN CONTENT OF WHOLE SALIVA OF CARIES SUSCEPTIBLE AND CARIES INSUSCEPTIBLE INDIVIDUALS (EXPRESSED AS MILLIGRAMS PER CENT)

Nitrogen components	Z.A.	B.E.	C.H.	C.D.Y.	E.C.	F.C.
Ammonia N	11·4±0·84	10·6±0·73	7·1±0·51	4·0±0·33	7·9±0·49	5·9±0·53
Amino N	3·4±0·23	1·9±0·13	3·1±0·19	1·6±0·11	1·6±0·14	1·5±0·16
Total N	93·4±6·6	73·8±4·2	82·3±4·5	59·0±3·1	50·0±3·1	49·9±3·4

Each value represents the mean of ten analyses on 10 separate days, with its standard error.

DISCUSSION

In the present investigation under relatively standardized conditions, eighteen free amino acids of whole saliva and of the secretions of the salivary glands have been investigated in three individuals. Likewise, studies were made of the ammonia nitrogen, amino nitrogen and total nitrogen of the whole saliva of six individuals.

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While a limited number of individuals were examined in each category, the analyses were repeated sufficiently over a long enough period of time to permit statistical evaluation and comparison of the results. With regard to incidence of amino acids, some, such as aspartic and glutamic acids, threonine, serine, glycine, alanine, and the combined group of phenylalanine, leucine and isoleucine, were detected regularly in whole salivas. There was considerable variation in proline, cystine, valine, methionine, tyrosine, tryptophan, histidine, lysine and arginine. The total free amino acids in the whole salivas analysed quantitatively ranged from $3{\cdot}44 \pm 0{\cdot}40$ to $4{\cdot}78 \pm 0{\cdot}31$ mg per cent. The free amino acids in the submaxillary secretions composed 31·6, 30·1 and 48·7 per cent of the amino acids of the whole saliva of the three individuals used in the earlier determination of the free amino acids of whole saliva. The submaxillary secretions, at the time of analysis, had not been subject, even briefly, to the enzymatic activity of the oral bacterial flora. The secretion of the parotid glands was found to contain few amino acids and even these were present in very low concentration. An analysis of the secretion of the sublingual gland was not made, for it has not been possible to obtain sufficient amounts of this fluid under circumstances in which it could be designated unequivocally as sublingual secretion.

In studies of the nitrogenous components of saliva, it was found that salivary ammonia nitrogen differed in 80 per cent, total nitrogen differed in 70 per cent and amino nitrogen differed significantly in slightly more than 53 per cent of all possible comparisons between the six individuals. These findings correspond, in general, to the relatively wide fluctuations in the free amino acid contents of salivas.

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THE CARBOHYDRATE CONSTITUENTS OF HUMAN SALIVA

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Abstract—Four preparations of pure salivary mucoid were prepared from pooled samples of activated saliva. The nitrogen, hexosamine, fucose, "true" hexose and sialic acid contents were estimated in each sample. In addition, chromatograms were run in order to examine the monosaccharide and hexosamine constituents. Two preparations were examined by means of ultraviolet light at wavelengths from 235 to 320 mμ. The results were essentially in agreement with those of BERGGÅRD and WERNER in that the preparations consisted predominantly of fucomucin, the sialic acid content being quite low. The possible role of salivary mucoid in the caries process is assessed.

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INTRODUCTION

STUDIES of some of the constituents of saliva are difficult because of their low concentration. However, whilst it is important to be aware of the composition of any biological material, it is all the more important should certain parts of that material be possibly implicated in disease processes. The part played by salivary mucoid as a factor in the initiation of dental caries is not known but it has been suggested as being a possible disruptive force because of its carbohydrate content and possible cation binding power (KNOX and STILL, 1953; SULLIVAN and MCCOOMBE, 1961).

WERNER (1953) had reported on the constituents of glycoproteins isolated from mucous epithelium and epithelial secretions, but it was not until 1958 that BERGGÅRD and WERNER described the carbohydrate constituents of human saliva. This paper reports our investigations into the composition of salivary mucoid.

MATERIALS AND METHODS

Materials

Saliva. Samples of paraffin-stimulated saliva were collected from members of undergraduate classes and pooled.

Salivary mucoid. Preparations of salivary mucoid were made by a modification of the method described by CURTAIN and PYE (1955). The pooled saliva was lightly centrifuged to remove debris. The supernatant was then adjusted to pH 4 using 5 N acetic acid and again centrifuged for 15 min, the supernatant being retained and neutralized with 5 N NaOH. This solution was then dialysed for 3 days against two changes of distilled water. The solution was made 0·1 M with respect to barium, using solid barium acetate after which methanol, precooled to −40°C, was added with stirring to give an alcohol concentration of 64% (v/v). After centrifugation the

precipitate was dissolved in 0·1 M solution of ethylene-diamine tetra-acetic acid (disodium salt) and dialysed for 60 hr against three changes of distilled water. The solution was then refractionated under identical conditions except that the methanol concentration used was 56%. The final preparation was obtained by freeze-drying, yielding a creamy-white flaky substance.

Methods

In order to obtain results comparable with those of BERGGÅRD and WERNER (1958) similar analytic methods were used when possible, such methods having been chosen as those most suitable for use in relation to the estimation of this material.

Nitrogen was determined by the micro-Kjeldahl method (MCKENZIE and WALLACE, 1954).

Hexosamines were determined by the Morgan–Elson method as modified by RONDLE and MORGAN (1955).

Fucose was determined by the method of GIBBONS (1955).

The "true" hexose content was obtained by subtracting the fucose figure from the "total" hexose value, obtained by the Vasseur method (VASSEUR, 1948).

Sialic acid was determined by the Bial method as described by BLIX, SVENNERHOLM and WERNER (1952). A sample of methoxy sialic acid kindly supplied by Dr. G. L. ADA of the Walter and Eliza Hall Institute of Medical Research, Melbourne, was used as a standard. The monosaccharide and hexosamine constituents were assayed qualitatively by descending paper partition chromatography using butanol–pyridine–water (6:4:3) as a solvent (JEANES, WISE and DIMLER, 1951). Para-anisidine hydrochloride was used as a spray reagent for monosaccharides (HOUGH, JONES and WADMAN, 1950) whilst the hexosamines were sprayed with alkaline acetyl acetone–butanol followed by Ehrlich's solution (PARTRIDGE, 1948).

Three preparations were also analysed for the presence of aromatic amino-substances, dilute solutions being examined spectroscopically with ultra-violet light through wavelengths from 235 to 320 mμ.

RESULTS

Four preparations of salivary mucoid were made. The first, which was not refractionated, yielded 91 mg per 100 ml saliva. The remaining three refractionated preparations yielded 61, 56 and 34 mg per 100 ml respectively. All were soluble in water to approximately 0·5 g per 100 ml but they were most readily soluble in alkaline solutions. The analysis of each specimen is set out in Table 1, which includes for comparison the mean values of seven individual preparations made by BERGGÅRD and WERNER (1958). Fig. 1 contains two curves which are representative of the optical density curves obtained when aqueous solutions were examined by ultra-violet light.

DISCUSSION

In 1953 WERNER reported the results of studies on the glycoproteins of mucous epithelium and secretions from a number of organs, a survey which was subsequently extended to include saliva (BERGGÅRD and WERNER, 1958). He reached the conclusion

TABLE 1. THE PERCENTAGE CONCENTRATION OF THE NITROGEN AND CARBOHYDRATE COMPONENTS OF FOUR PREPARATIONS OF SALIVARY MUCOID

Preparation	Nitrogen	Hexosamine	Fucose	"True" hexose	Sialic acid	Chromatograms
1	10·1	5·0	2·2	7·08	1·0	Galactose (faint trace) Galactosamine (faint trace)
2	9·1	5·0	2·15	7·05	0·5	Galactose (trace) Galactosamine Glucosamine (trace)
3	9·7	8·3	3·0	13·5	0·8	Galactose Mannose and/or fucose Glucosamine and/or galactosamine
4	10·5	9·0	3·3	14·0	0·9	Galactose Fucose Galactosamine
Mean	9·9	6·8	2·7	10·3	0·8	
Mean obtained by BERGGÅRD and WERNER (1958)	10·9	6·0	3·3	8·0	2·1	Galactose Fucose Mannose (trace)

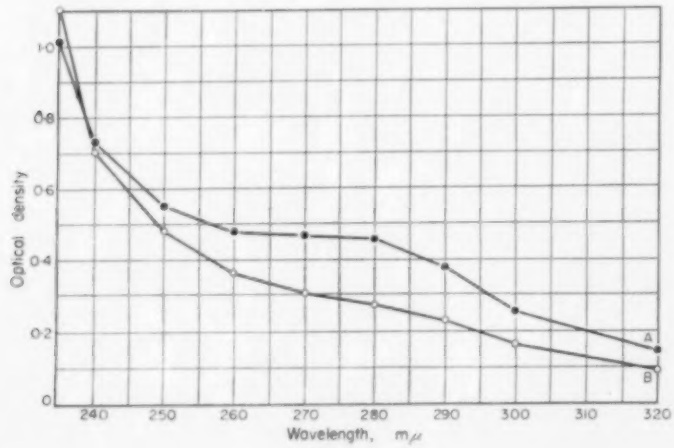

FIG. 1. The examination of two preparations of different nitrogen concentration by means of ultra-violet light at wavelengths from 235 to 320 mμ.

that secretions contain a mixture of glycoproteins of different composition with respect to their carbohydrate components. The predominant product referred to as fucomucin contains fucose, galactose, glucosamine and galactosamine—the components of the blood-group substances. The second major glycoprotein, called sialoprotein and first obtained from submaxillary gland by BLIX in 1936, contains sialic acid and galactosamine in equimolar proportions. The strict division of glycoproteins in these two categories cannot be generally applied, however. GIBBONS (1959) has described a "blood-group like" substance of animal origin containing 14 per cent sialic acid, and PUTZAI and MORGAN (1960) obtained a preparation of blood-group specific Le^a substance with 19 per cent sialic acid.

In agreement with the results of BERGGÅRD and WERNER (1958) the preparations of salivary mucoid described in this paper consist predominantly of fucomucin; the sialic acid content is even lower than that found by the Scandinavian investigators, a difference probably brought about by the different methods of isolation. Although analyses for carbohydrate components provide the most convenient method for comparing glycoproteins, the carbohydrate moiety is the minor component, the amino-acids making up the major part of the complex. Typically the glycoproteins lack aromatic amino-acids and the ultra-violet absorption curve for a preparation containing 9·1 per cent nitrogen supports this conclusion (Curve B, Fig. 1). Another preparation contained 10·5 per cent nitrogen and the presence of contaminating protein is indicated by the inflexion at 280 mμ (Curve A, Fig. 1).

Examination of Table 1 would indicate that salivary mucoid contains about 10 per cent hexose and 1 per cent sialic acid. The likelihood of salivary mucoid being a source of carbohydrate for acidogenic organisms is not great. It is possible, however, that free calcium ions could become bound to the carboxyl group of free sialic acid, particularly in areas where mucoid is concentrated, as is assumed to occur in plaques.

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EXPERIMENTAL REPLANTATION OF TEETH IN DOGS AND MONKEYS

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Abstract—Fifty-eight teeth were replanted in four monkeys and six dogs. Thirteen teeth were replanted without periodontal membrane, fifteen were dried in air for varying periods of time, and thirty were re-inserted into the alveolus immediately following extraction. Observation periods varied between 8 days and 33 months. Teeth replanted without periodontal membrane never regained a normal attachment apparatus. At first the periodontal space was occupied by soft tissue. This was soon replaced by bone and the establishment of ankylosis was accomplished within 30 days. A normal periodontal membrane was never found in the teeth replanted with a dried periodontal membrane, although the teeth that were dried for short periods exhibited minor areas of normal periodontal attachment. In teeth replanted with vital periodontal membranes, the periodontal membrane was always reformed after replantation. Ankylosis was never observed. Success or failure in replantation seems greatly to depend on the presence or absence of a vital periodontal membrane. The hypothesis is offered that the epithelial remnants of Malassez play a role in the maintenance of the periodontal space.

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TOOTH replantation has been the subject of interest for centuries, and innumerable case reports have been published. However, experimental investigations on the healing processes in the periodontal membrane after such procedures are relatively scarce.

For 200 years one of the main points of discussion has been whether the teeth should be replanted with or without the periodontal membrane fibres which remain attached to the tooth after extraction. As early as 1755, HUNTER reached the conclusion that a vital periodontal membrane was a prerequisite for a successful reunion. The same opinion was expressed by YOUNGER in 1886.

More or less extensive root resorption has been a consistent finding in experimental replantation. On the basis of animal experiments, FREDEL (1886) and SCHEFF (1890) suggested that root resorption does not occur when teeth are replanted with their periodontal membranes. WILKINSON (1917), on the other hand, found that the presence or absence of periodontal membrane had no appreciable effect, as resorption took place to some extent in either case. These discrepancies might be explained by the inadequate investigation techniques of that time. However, similar disagreement is encountered in recent publications as well. Thus, HEISS (1944) contended that the periodontal membrane is of little or no importance, and may even be detrimental to regeneration, whereas BÖDECKER and LEFKOWITZ (1935) and HAMMER (1934, 1937) were of the opinion that it is absolutely necessary for healing.

Various devices have been advocated in order to secure vitality of the periodontal membrane during the extra-oral handling (KRÖMER, 1948).

There seems to be no disagreement as to the necessity of replacing the pulp with a proper root filling before re-implantation (PINDBORG and HANSEN, 1951).

The purpose of the present study was to gain more information on the healing processes concerned with replantation and on the importance of the presence or absence of periodontal membrane remnants. In the case of replantation with periodontal membrane it was necessary to distinguish between a vital and a non-vital membrane. It was thus possible to evaluate the importance of the periodontal membrane as an entity and also to study the role played by its specific cellular and fibrous elements for the maintenance of normal function.

MATERIAL AND METHODS

Fifty-eight teeth from four monkeys and six dogs were used for the present investigation; all the animals were in good health. The experimental procedures were carried out under Nembutal anaesthesia. The teeth were extracted in the usual manner, but special care was taken during the extraction in order to avoid unnecessary damage to the supporting structures. Three different techniques were employed.

Series I: *Replantation without periodontal membrane* (*Thirteen teeth*, *Table* 1)

Subsequent to extraction, extirpation of the pulp was carried out extra-orally. After proper root-filling with chloroform–guttapercha paste and guttapercha points, the periodontal membrane was removed by means of suitable hand instruments or rotating rubber wheels.

TABLE 1. SERIES I: THIRTEEN TEETH REPLANTED WITHOUT PERIODONTAL MEMBRANE

Animal	Tooth	Observation period (days)
Monkey 16	Upper right first incisor	29
	Upper left first incisor	29
	Lower right first incisor	33
	Lower left first incisor	33
Monkey 15	Upper right first incisor	81
	Upper left first incisor	81
	Lower right second incisor	85
	Lower left second incisor	85
Monkey 14	Lower right first incisor	85
	Lower left first incisor	85
	Upper right first incisor	85
	Upper left first incisor	85
Monkey 13	Upper left first premolar	85

Series II: *Replantation with air-dried periodontal membrane* (*Fifteen teeth, Table* 2)

The teeth were root-filled extra-orally according to the procedure described in Series I, after which they were allowed to dry in air for from 15 min to 2 hr. Thereafter the teeth were replanted.

TABLE 2. SERIES II: FIFTEEN TEETH REPLANTED WITH DRIED PERIODONTAL MEMBRANE

Animal	Tooth	Drying period (min)	Observation period (days)
Dog 20	Lower right first incisor	45	8
	Lower left first incisor	45	8
	Upper right second incisor	45	8
	Lower right third incisor	45	15
	Lower left third incisor	45	15
Dog 21	Lower right third molar	45	33
	Lower left third molar	45	33
	Lower left second incisor	45	33
	Lower left third incisor	45	33
Monkey 13	Lower right first premolar	30	42
	Lower left first premolar	30	42
Dog 22	Upper right first incisor	45	56
	Upper left third incisor	45	56
Monkey 14	Upper right first premolar	120	42
	Lower left first premolar	15	85

Series III: *Replantation with vital periodontal membrane* (*Thirty teeth, Table* 3)

In this series every effort was made to keep the periodontal membrane vital. Prior to extraction a hole was drilled through the buccal surface of the crown into the pulp chamber. Immediately after the extraction the tooth was re-inserted into the alveolus and kept there during the extirpation of the pulp and the subsequent root-filling. The root canals were in some cases filled to the apex, whereas in others only the coronal part was filled, the larger parts of the root canals being left open. In all cases the tooth stayed outside the socket only for a few seconds altogether.

Most of the replanted teeth were fixed to neighbouring teeth with thin steel ligatures. After varying periods of time the wires were removed. A few premolars in monkeys, however, were put back into the socket without fixation.

Due to the circumstances, the operations could not be performed under aseptic conditions. In most cases, however, the roots were washed in 1% phenyl mercuric acetate before re-insertion into the alveolus. The animals were fed an adequate diet during the experimental period; no steps were taken to limit the chewing of hard food.

TABLE 3. SERIES II: THIRTY TEETH REPLANTED WITH VITAL PERIODONTAL MEMBRANE

Animal	Tooth	Observation period (days)
Dog 29	Upper right second premolar	30
	Lower right third molar	30
	Lower right second premolar	30
Dog 28	Lower right third molar	31
	Lower left third molar	31
Dog 29	Upper right third incisor	42
	Upper left first premolar	42
	Upper left second premolar	42
	Lower left third molar	42
	Lower left second incisor	42
	Lower left third incisor	42
	Lower left second premolar	42
	Lower left third premolar	42
	Lower right second incisor	51
	Lower right third incisor	51
	Upper right first incisor	51
Dog 28	Upper left second incisor	60
	Upper left first premolar	60
	Lower left third incisor	60
	Lower left first premolar	60
	Lower right first premolar	70
	Lower right third incisor	70
	Lower right second incisor	70
	Lower left first incisor	70
	Upper right second incisor	82
	Upper right first incisor	82
Dog 26	Lower right second incisor	988
	Upper left second incisor	1000
	Lower left first incisor	1019
	Lower left second incisor	1019

The experimental periods varied between 8 days and 33 months (Tables 1, 2, 3). At the end of the experiment, the ages of the dogs were 7, 8, 10, 12, 13 and 42 months. In the monkeys the experiment was started shortly after the eruption of all the permanent teeth.

When the animals had been killed, the jaws were carefully taken out and immediately immersed in 10% neutral formalin. After decalcification in 5% nitric acid, the specimens were embedded in paraffin or celloidin and sectioned in the bucco-lingual or mesio-distal direction. The sections were stained with haematoxylin and eosin, and with Mallory's connective tissue stain.

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FINDINGS

Histologic examination showed that the healing followed certain patterns which were closely related to the three experimental procedures. For this reason, the findings will be described separately for each of these groups.

Series I: *Replantation without periodontal membrane*

The teeth belonging to this category never regained a normal attachment apparatus. The removal of the periodontal membrane by hand or by rotating rubber wheel did not seem to affect the final result. In either case, no remains of the original periodontal membrane was found. After less than 30 days, a bony attachment was established between the replanted teeth and the jaws to a point shortly apical to the epithelial cuff (Figs. 1, 2, 3). Small areas of active osteoclastic resorption were scattered at random along the root. Howship's lacunae with osteoclasts were frequently located close to areas of bone tissue formation (Figs. 1 A, B, C, 3 A). Resorption was observed almost without exception in the gingival third of the roots (Figs. 1 E, 3 A). Areas indicating previous resorption of the root were filled with bone tissue (Figs. 1, 3). In some cases the roots were reduced in length and width due to resorption (Fig. 2). In others, most of the root surface was still covered with intact cementum (Fig. 3). The periodontal membrane was occasionally replaced by fat tissue (Fig. 3 B).

Series II: *Replantation with air-dried periodontal membrane*

To some extent the histologic picture in Series II varied with the length of the extra-oral interval. A normal periodontal membrane was never found. After drying for 2 hr, the periodontal space was widened. Numerous Howship's lacunae with osteoclasts indicated that resorption was going on along the entire root (Fig. 4 A). Young connective tissue cells and fibres without functional orientation constituted the bulk of the soft tissue.

In limited areas bone tissue had formed directly upon the replanted root (Fig. 4 B). It was evident, however, that the formation of bone started from the alveolar side of the periodontal space and not from the tooth. This was more frequently seen in teeth that had been lying in the air for shorter periods of time (Figs. 5, 6). Bone trabeculae in the process of bridging the periodontal space (Figs. 5 D, 6 B) or smaller areas of ankylosis (Fig. 5 B) were encountered along the entire intra-alveolar part of the root.

In teeth that had been dried for 30 or 15 min, small parts of the periodontal membrane were normal or close to normal; no resorption or bone formation took place here, and the periodontal fibres revealed functional orientation (Figs. 5 A, 6 A, 7 A, C). In all these places epithelial remnants of Malassez were observed between the fibre bundles. The supra-alveolar periodontal membrane in these teeth was in most cases normal (Figs. 5 A, E, 6 A, E). Usually the deepest point of the epithelial cuff was located at the cemento–enamel junction.

In Fig. 7 a tooth is shown whose buccal root fractured during the extraction. The lingual root with the crown was, however, replanted. The fractured root maintained a normal periodontal membrane (E, D). The lingual root was not root-filled. Therefore, the pulp died and a granuloma developed (B). The periodontal membrane

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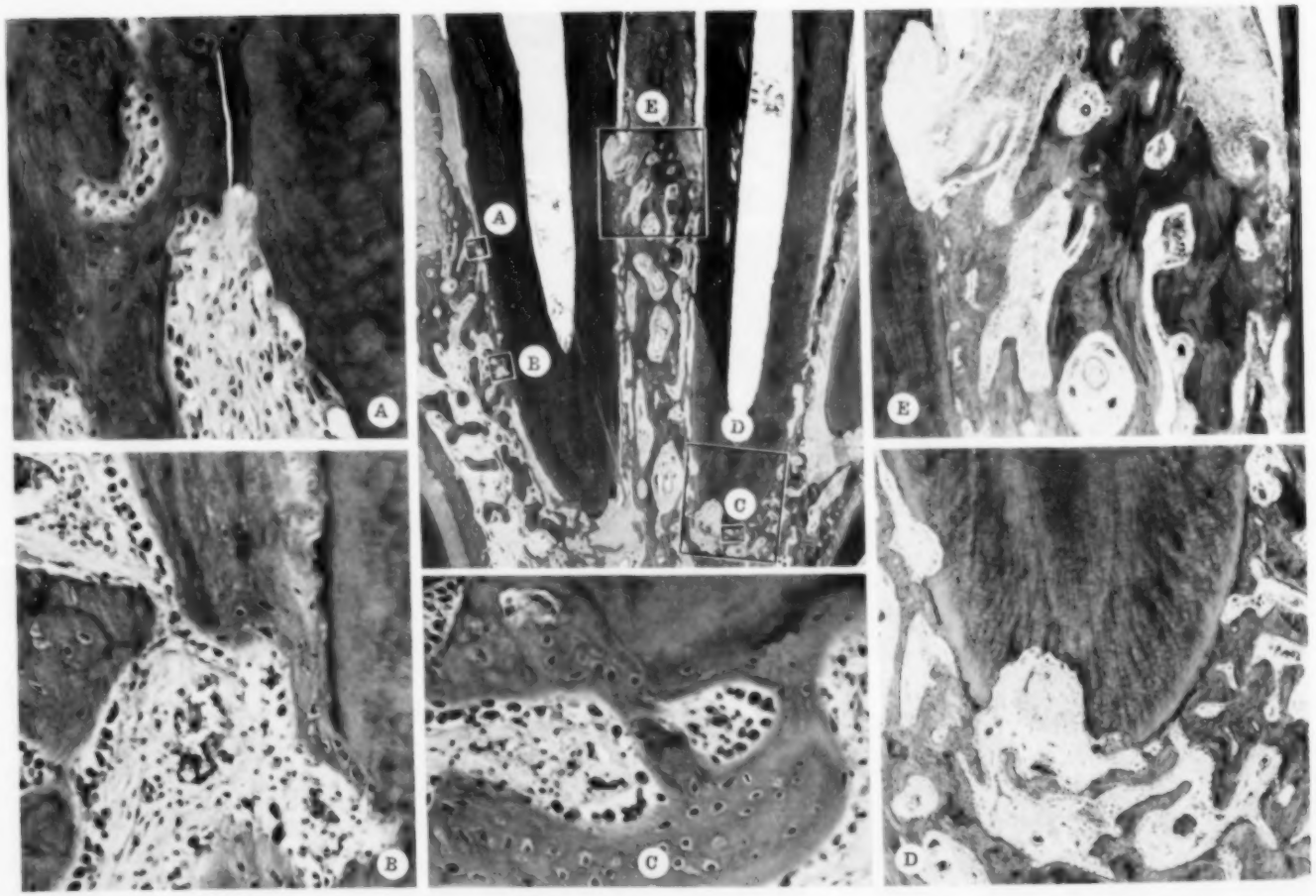

FIG. 1. Monkey 16. Upper central incisors 29 days after replantation without periodontal membrane. Ankylosis established (A, B, C, D, E). Root resorption shortly apical to epithelial cuff (E). Haematoxylin and eosin.

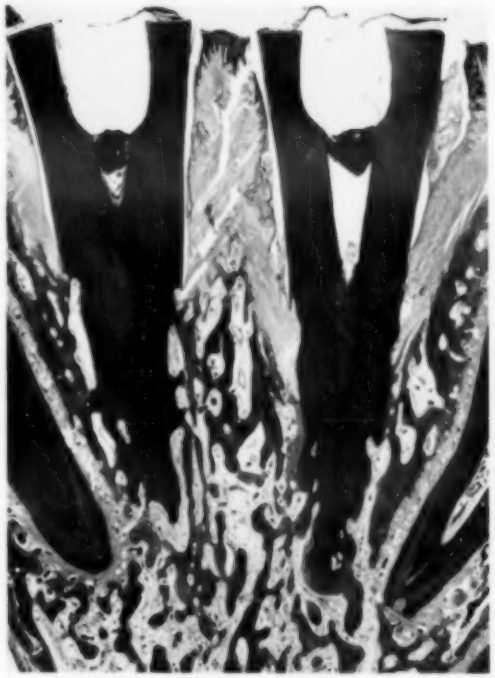

FIG. 2. Monkey 14. Lower central incisors 85 days after replantation without periodontal membrane. Roots reduced due to extensive resorption. Formation of new hard tissue upon dentine. Haematoxylin and eosin.

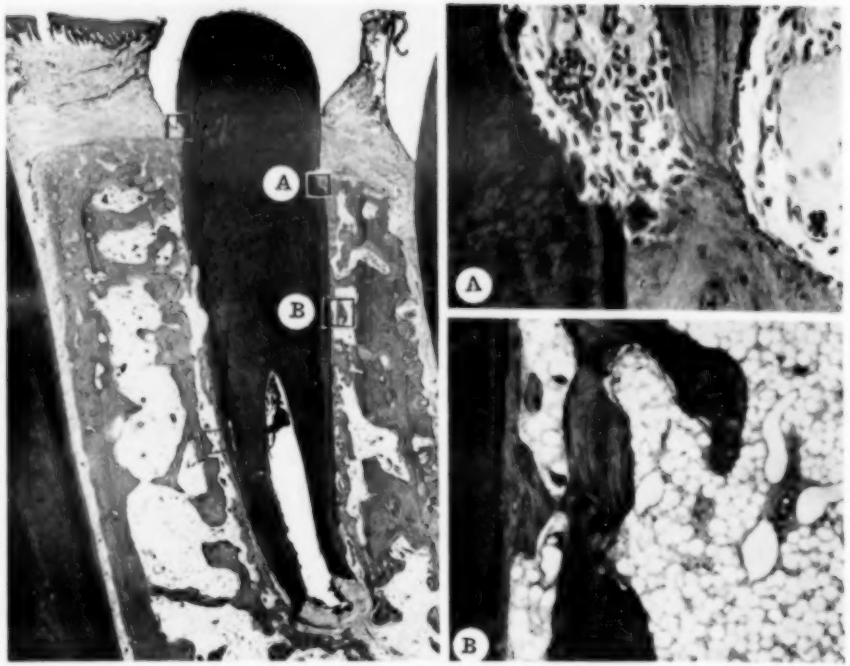

FIG. 3. Monkey 13. Upper left premolar 85 days after replantation without periodontal membrane. Root resorption in the gingival third (A). The largest parts of root covered with cementum upon which bone tissue is laid down. Periodontal membrane replaced by fat (B). Haematoxylin and eosin.

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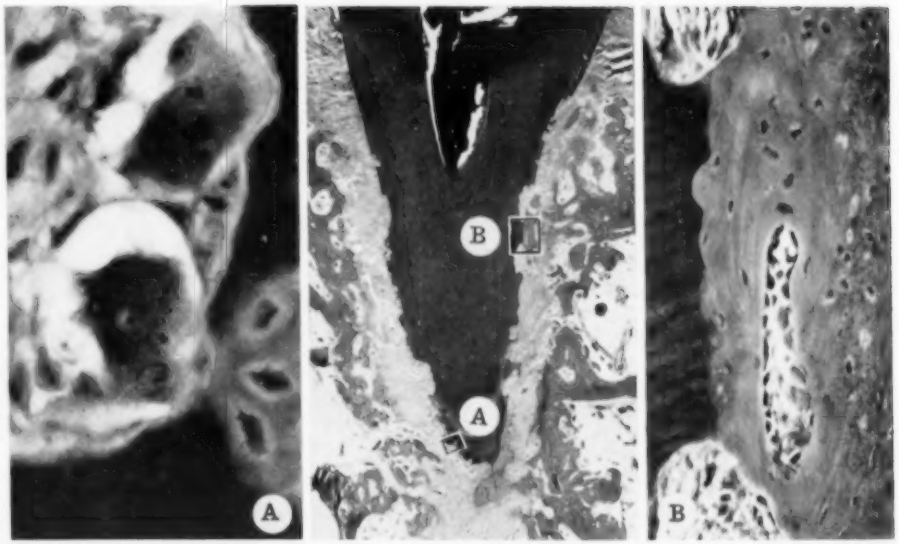

FIG. 4. Monkey 14. Upper right premolar dried in air for 2 hr prior to replantation. Observation period 42 days. Widened periodontal space. Active root resorption along entire root (A). Small area of ankylosis (B). Haematoxylin and eosin.

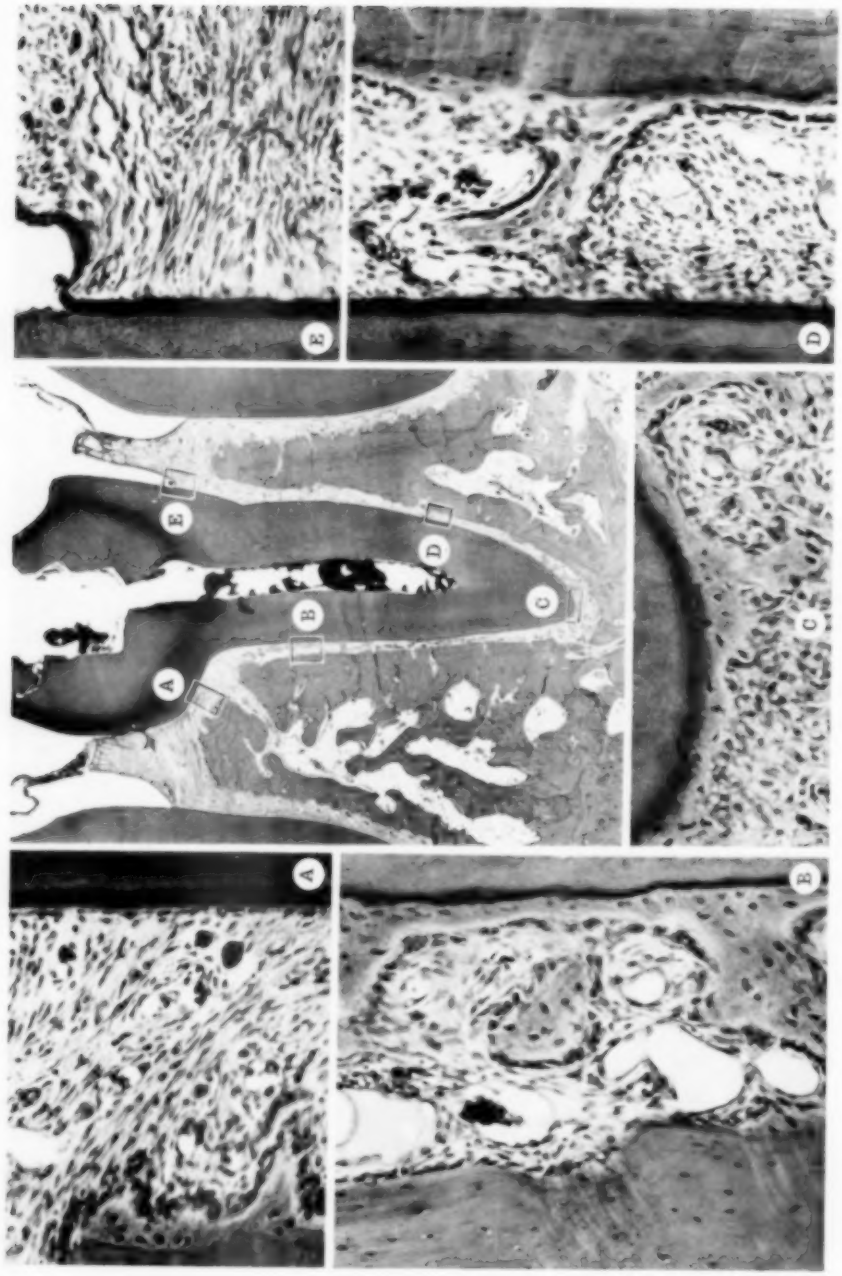

FIG. 5. Monkey 13. Distal root of lower left premolar dried in air for 30 min. Observation period 42 days. Small areas of normal periodontal membrane (A). Epithelial cuff ends at CEJ (E). Bone trabeculae forming from the alveolar bone (D) and area of ankylosis (B, C). Haematoxylin and eosin.

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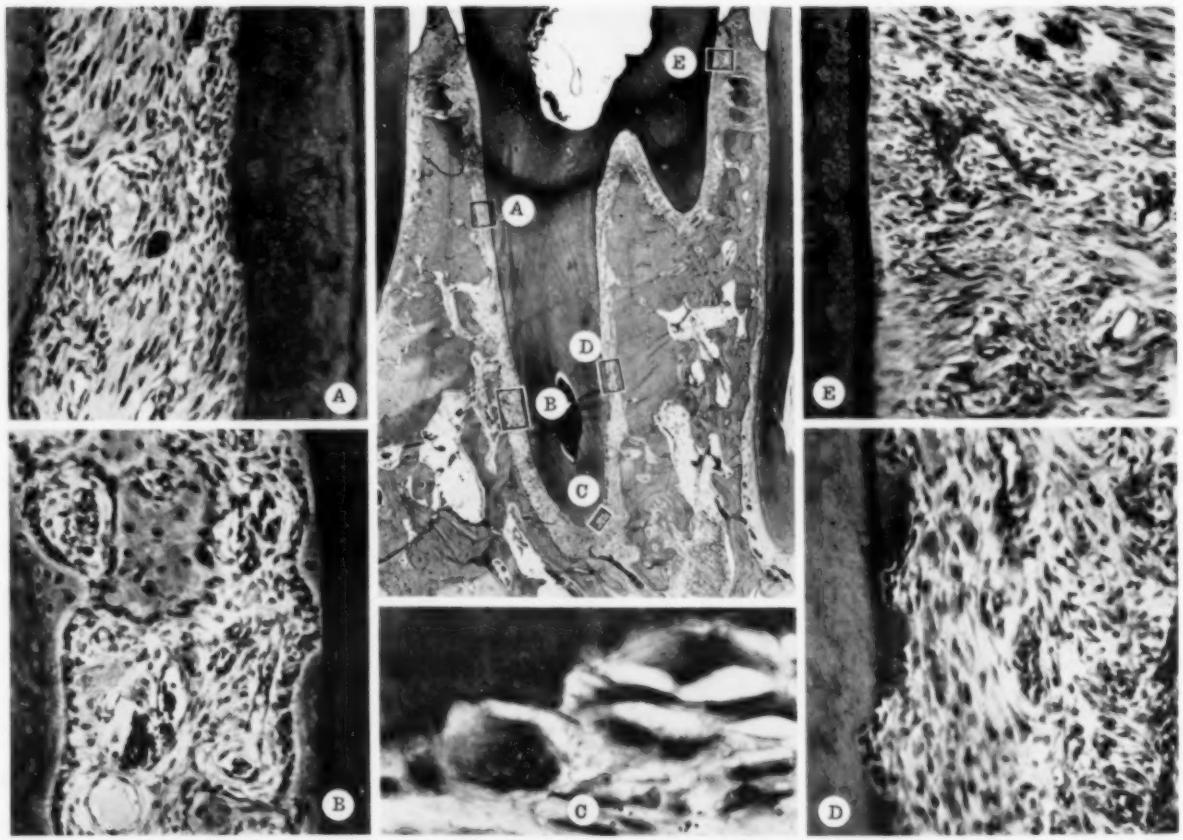

FIG. 6. Mesial root of the premolar shown in Fig. 5. Supra-alveolar connective tissue showing functionally oriented fibres (E). Small area of normal periodontal membrane with epithelial rests of Malassez (A). Areas of resorption in the intra-alveolar part of root (C, D). Area of ankylosis (B). Haematoxylin and eosin.

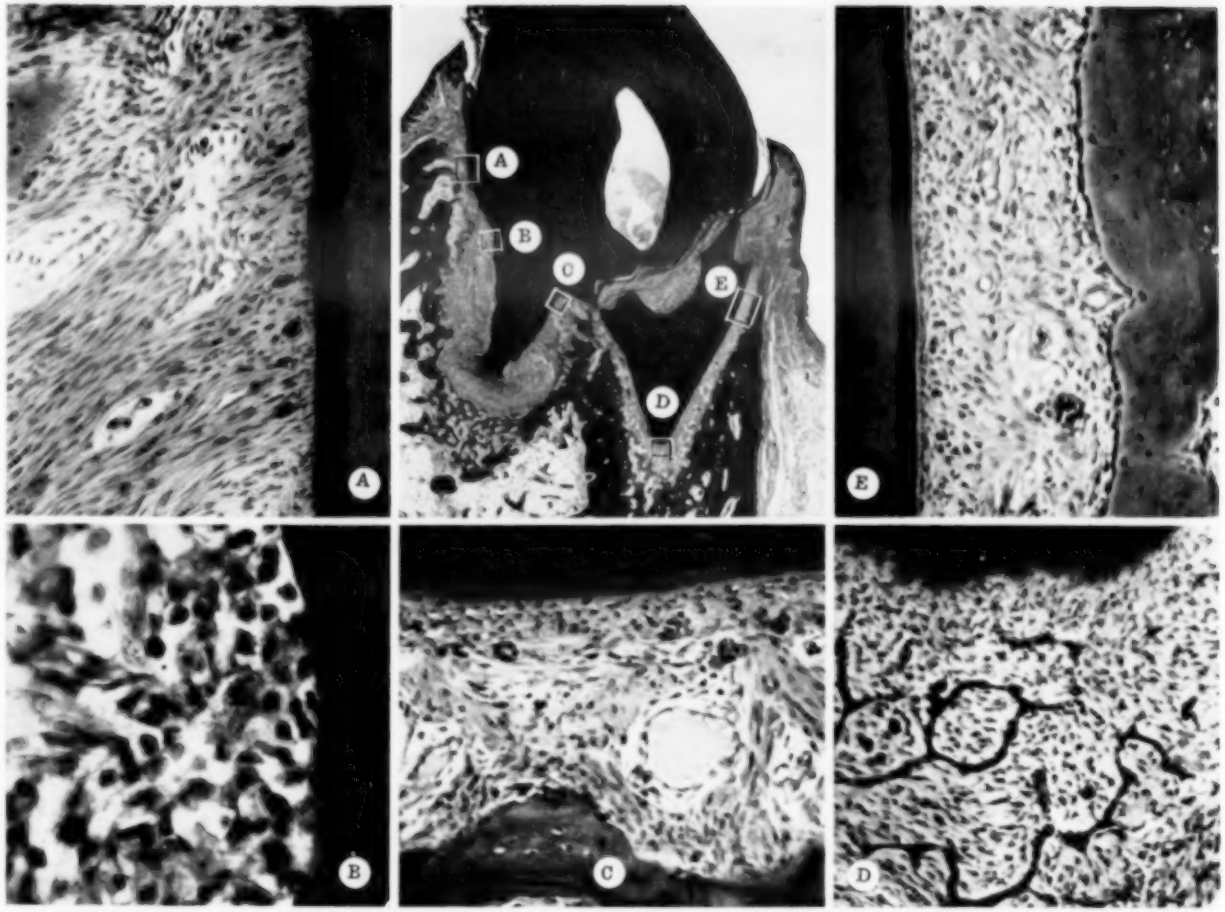

FIG. 7. Monkey 14. Lower left premolar. Buccal root fractured during extraction. Lingual root dried in air for 15 min prior to replantation. Observation period 85 days. Fractured root revealing a normal periodontal membrane (E). Tangential section showing epithelial network of Malassez (D). Severe inflammation around apical half of replanted root (B). Small areas of normal periodontal membrane with epithelial rests of Malassez (A, C). Haematoxylin and eosin.

PLATE 6

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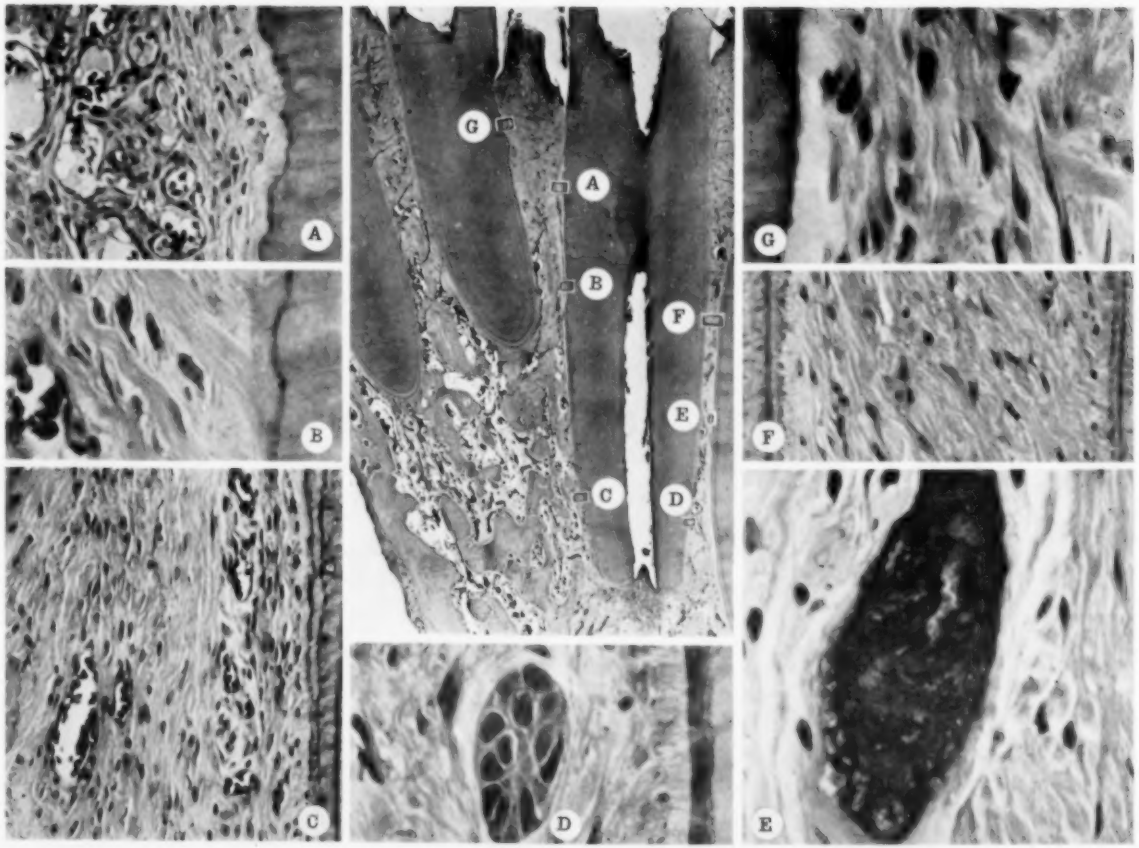

FIG. 8. Dog 29. Lower right lateral incisors 51 days after replantation with vital periodontal membrane. Resorption subsided and new Sharpey's fibres inserted into the cementoid (A, B, C). Occasionally areas of parallel fibre-orientation were seen (C). Interdental space too narrow for alveolar bone to be formed. Nevertheless, functionally oriented fibre bundles connecting the two roots (F). Epithelial rests of Malassez (D). Calcification of necrotized soft tissue elements at narrowest part of periodontal membrane (E). Haematoxylin and eosin.

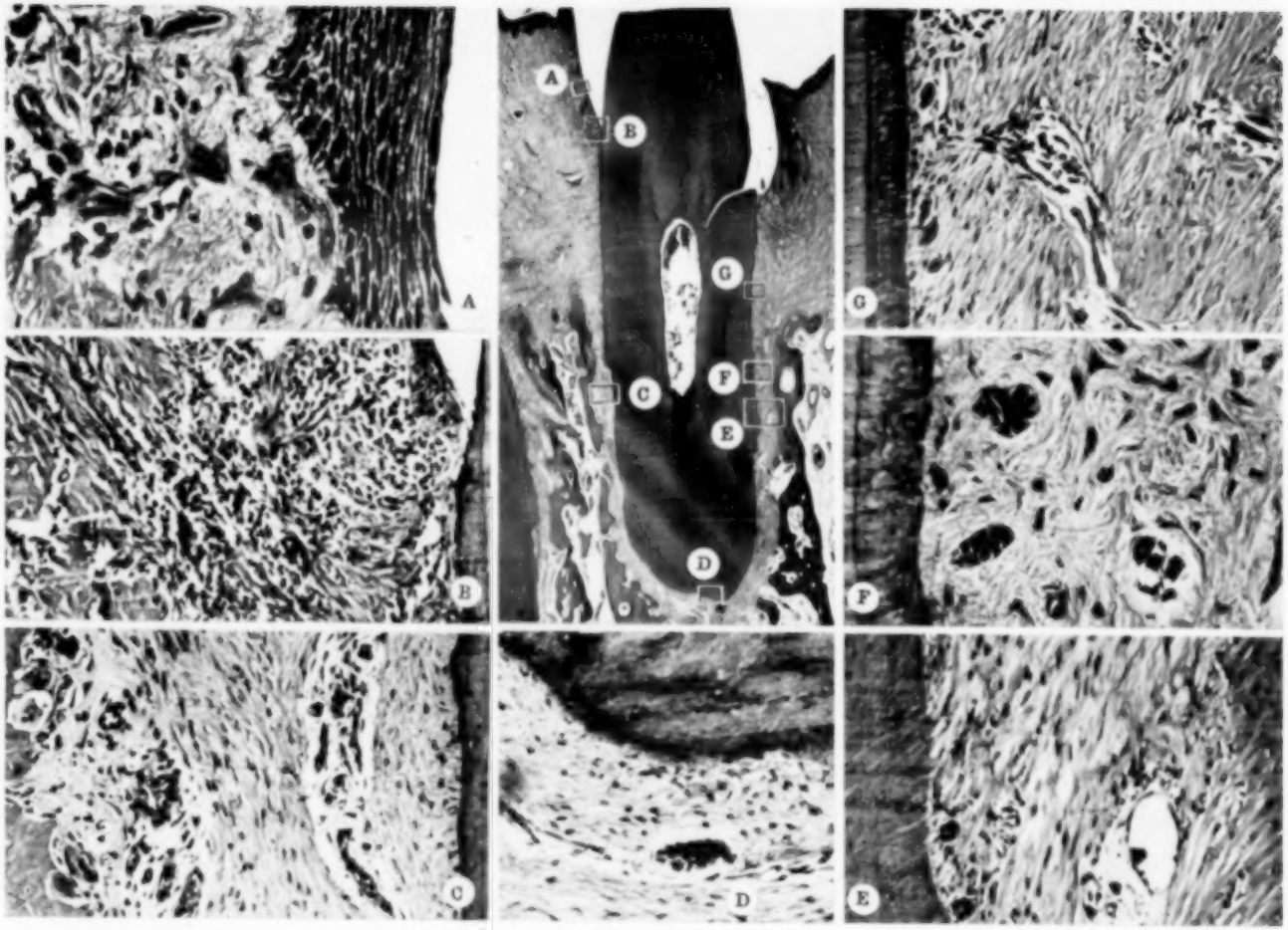

FIG. 9. Dog 26. Lower right lateral incisor 32 months after replantation with vital periodontal membrane. Normal width of periodontal space. Resorption lacunae filled in (C, D) and normal apposition of secondary cementum (E, F, G). Functional orientation of periodontal fibres (E, G). Presence of epithelial rests of Malassez (D, E, F, G). Fairly normal epithelial cuff (A) showing no proliferation beneath CEJ (B). Haematoxylin and eosin.

showed normal conditions only in two small areas, i.e. at the entrance of the alveolus on the lingual side and in the bifurcation. These areas represented the only places where epithelial rests could be found (A, C).

Series III: *Replantation with vital periodontal membrane*

In this series a periodontal membrane was always present. Ankylosis or a tendency of the bone to bridge the periodontal space was not discovered.

In the short term experiments the fibres were functionally oriented in most instances (Fig. 8 B, F), but fibre bundles parallel to the root surface were also seen (Fig. 8 C).

Almost all the teeth showed active or arrested resorption (Fig. 8 A, B, C). This was particularly the case in the gingival third (Fig. 8). Epithelial rests were commonly observed in the supra-alveolar as well as in the intra-alveolar periodontal membrane (Fig. 8 D). In the majority of cases the epithelial cuff ended at the cemento–enamel junction, and it did not differ from that of untreated neighbouring teeth.

Fig. 8 illustrates a case where two adjacent teeth were replanted. The right one has moved distally towards an untreated tooth. The periodontal membrane is rebuilt, but between these teeth there is no formation of new alveolar bone. Nevertheless, the soft tissue appears quite normal (F).

Although the periodontal membrane in some places showed healthy conditions after 30–80 days, the healing in these short term experiments appeared to be incomplete.

In the teeth which had been in function for almost 3 years after replantation (Fig. 9), the dynamic processes which characterized the short term specimens had subsided. The periodontal space was of normal width, and the fibres were functionally oriented (E, G). Epithelial rests were scattered throughout the periodontal membrane (D, E, F, G). Resorption lacunae were filled with secondary cementum (C, D). The epithelial cuff ended at the cemento–enamel junction (B). Inflammatory cells were observed in the subepithelial connective tissue. However, this local inflammation did not exceed that adjacent to the neighbouring untreated teeth. By and large, the periodontal membrane 33 months after replantation showed characteristics of normally functioning teeth.

DISCUSSION

The reported series may explain some of the discrepancies between earlier experiments on replantation. The reason may be found in the strictly differentiated experimental procedures and the length of the observation periods.

Ankylosis

Replantation without periodontal membrane and replantation after drying of the periodontal membrane had one major feature in common, i.e. the establishment of ankylosis. Initially, the blood clot is organized to granulation tissue, but this is soon replaced by bone. Although root resorption usually precedes the development of ankylosis, this is not a necessary stage. Bone forms from the wall of the alveolus

in the form of narrow bridges which eventually reach the root surface. These bone trabeculae in turn unite and form a solid plate directly upon the intact cementum or the previously resorbed cementum or dentine.

The main difference between the reaction around teeth replanted with dried periodontal membrane and that around teeth in which the periodontal membrane had been removed, was that the healing around the dried teeth lagged one step behind as far as time is concerned. The final results were identical. Besides that, there were found limited areas of normal periodontal membrane around the dried teeth. This will be discussed later. Ankylosis may be established 30 days after replantation. There may be absence of pathologic conditions, although functional normality is not established. From a histopathological point of view this must be characterized as a complete healing. However, such an attachment does not serve the requirement of a functioning tooth. This is most likely the reason why an ankylosis does not remain unchanged. During function the teeth have no longer a soft tissue cushion to subdue the masticatory forces. Consequently, in teeth with moderate function resorption will start, giving as a result a soft tissue space between the root and the surrounding bone. However, this space is not maintained and new bone is formed in contact with the root surface. The joint effect of resorption and bone formation eventually leads to the replacement of the entire root by bone. In cases of increased functional stress such teeth are well known to be liable to fracture.

Effect of vital periodontal membrane

The most striking finding in Series III was the absence of ankylosis. Without exception connective tissue filled the space between the root and the alveolar bone. Parts of the periodontal membrane appeared normal within 30 days, whereas healing might not be completed even after 3 months. In isolated areas along the root surface resorption was still going on. Apposition of cementum was also found during this period, but formation of bone bridges across the periodontal space never occurred.

Hammer (1934, 1937) suggested that the centres of resorption and apposition would lend themselves to ankylosis. In his opinion resorption starts where the periodontal membrane is damaged during extraction. Indeed, it is impossible to remove a tooth without damaging the root surface in small areas, and it may well be that these areas give rise to resorption. This was seen buccally and lingually in the gingival third of nearly all replanted teeth of the present investigation. The long term experiments, however, definitely show that root resorption is not necessarily followed by ankylosis. Nearly 3 years after replantation teeth with a vital periodontal membrane revealed complete healing and no ankylosis.

The long term experiment clearly shows that it is possible for a normal periodontal membrane to become re-established after replantation, and it also indicates that this attachment apparatus is not of an inferior quality and that permanent results may be achieved under favourable conditions.

Maintenance of the periodontal space

In Series I and II the periodontal space was filled with connective tissue for a short period of time prior to the establishment of ankylosis. The cells and fibres of

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this connective tissue did not differ from those of the original periodontal membrane. In spite of that, it was incapable of organizing itself into a functioning periodontal membrane and to maintain its normal width. Something has been lost with the original periodontal membrane, something that possessed the capacity of preventing fusion between the root and the bone. This seems to be the crucial point in the technique of replantation.

Normal conditions were found only in small areas of the periodontal membrane of teeth that had been dried for 15–30 min. In these areas the fibres were normally oriented between root and bone as in the teeth replanted with vital periodontal membrane. The connective tissue cells and fibres of the normal areas did not differ individually from those of neighbouring areas in which fibre orientation was absent. The main difference was the presence of epithelial remnants of Malassez in the normal parts. Thus, it must be assumed that the periodontal membrane had not become necrotic in all places in the teeth which had been dried for shorter periods and some of the epithelial remnants must have survived here. After 2 hr of drying the necrosis was apparently complete as areas of normal periodontal membrane were not observed. The epithelial remnants of Malassez were also always found in the teeth replanted with vital periodontal membrane.

It is remarkable that normal periodontal membrane was found only in the places where epithelial rests were present. The function of these cells is not fully understood. Most authors (GOTTLIEB, 1921; FISCHER, 1923; HIGAKI, 1932) are of the opinion that the epithelial remnants of Malassez have no particular function. ROBINSOHN (1926) suggested that they produced a hormone which prevents the fusion of cementum and alveolar bone. From his study on C-avitaminosis in monkeys WAERHAUG (1958) inferred that the epithelial rests may exert some protective activity against root resorption.

Root resorption was a common feature of all three experimental series, and it was most pronounced in the period shortly after replantation. In Series III root resorption was brought to a standstill after some weeks, whereas in Series I and II the resorption went on continuously. Probably it would eventually have effected the loss of these teeth. The possibility must be considered that the epithelial rests of Malassez may be a factor in the limitation of root resorption in teeth replanted with vital periodontal membrane.

Whether the epithelial rests are present because there exists a normal periodontal membrane, or the normal periodontal membrane is re-established due to the presence of the epithelial rests, cannot be fully understood from this material. However, it indicates that these cells may—perhaps just by their presence—play a role in the maintenance of the periodontal membrane.

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EFFECTS OF VARIOUS SUCROSE : CASEIN RATIOS IN PURIFIED DIETS ON THE TEETH AND SUPPORTING STRUCTURES OF RATS

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Abstract—Purified diets of varying carbohydrate: casein ratios were fed to female rats of the Harvard caries-susceptible strain for a stabilizing period of 28 days and then throughout pregnancy and lactation. Offspring of females fed a low-protein:high-sucrose diet grew more slowly and their molar teeth erupted later than rats born to females fed a control diet with a normal protein content. The molars of the offspring of the protein-deficient females were significantly smaller than those of the controls. This change in size was due to a decrease in the distance between the outer borders of the dentine rather than to a reduction in enamel thickness. Many of the third molars of these rats had missing cusps. The offspring of the deficient females had a significantly higher susceptibility to caries than the offspring of the control females.

Rats born to females fed a high-protein: low-sucrose diet had a tendency toward an increased susceptibility to a periodontal syndrome and were significantly less susceptible to dental caries than the offspring of females fed the control diet. There was also a slight tendency for a breakdown of the periodontal structures among the offspring of females fed the low-protein: high-sucrose diet, but the syndrome did not progress as rapidly as among the offspring of females on the high-protein: low-sucrose diet.

INTRODUCTION

MOST of the investigations into the effects of nutritional factors upon the teeth have been directed toward mineral and vitamin deficiencies. For the most part, the influences of the major components of the diet, especially the protein content, have been neglected. This study was directed toward a determination of the effects upon the oral tissues of gross changes in the carbohydrate: protein ratios imposed during pre-natal and early post-natal life, and also after weaning.

SCHWEIGERT *et al.* (1946a,b) and SHAW (1950) have shown that a substantial reduction in the incidence of dental caries in rats resulted when the majority of the sucrose in a purified diet was replaced by casein after the teeth had erupted. LOSEE *et al.* (1957) fed diets containing various casein: sucrose ratios to weanling rats for 6 weeks. The casein content of these diets varied between 20 and 40 per cent. They could find no differences in the concentrations of various components estimated quantitatively in whole teeth, nor in the incidence of dental caries. As the crowns of the molar teeth had, for the most part, already formed prior to the animals being transferred to the experimental diets, the inconclusive results were not unexpected.

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More recently, STACK and HOLLOWAY (1959) made a preliminary report on the carbohydrate levels in the enamel of rat molars which had developed while the animals were suckling from mothers fed diets containing different carbohydrate:protein ratios. The results suggested the possibility that variations in the chemical composition of the teeth had resulted from nutritional differences during development. BUXBAUM *et al.* (1957) and STEINMAN and HALEY (1957) have attempted to correlate the type and quantity of carbohydrate intake during development with subsequent caries-susceptibility and chemical composition of the teeth. None of the work has been entirely conclusive, but all has indicated the need for further investigation in this field.

EXPERIMENTAL METHOD

The experimental plan required the production of litters from protein-deficient rats. First it was necessary to decide on the lowest level of protein compatible with reproduction and the continued life of the offspring in the Harvard caries-susceptible strain. A pilot study was conducted using thirty-two 2-month-old female rats which were littermate distributed between four groups and fed purified diets P1, P2, P3 and P4, the composition of which differed only in the sucrose:casein ratio (Table 1).

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TABLE 1. COMPOSITION OF EXPERIMENTAL DIETS IN GRAMS

Ingredients	Diets				
	P1	P2	P3*	P4	P5
Sucrose	85	81	67	19	83
Crude casein	0	4	16	64	0
Vitaminized casein*	6†	6†	8	8	8
Vitaminized corn oil*	5	5	5	5	5
Salt mixture*	4	4	4	4	4

To every 100 g of each diet were added 15 g of Cellu flour and 2 g of dried whole liver substance.
* Based on diet 2700 (SHAW and GRIFFITHS, 1960).
† Specially prepared to contain same quantities of vitamins as in diets P3, P4 and P5.

Since reproduction on the lower levels of protein was expected to be poor, sixteen females were fed diet P1, eight diet P2, four diet P3 and four diet P4. They were maintained on these diets for 28 days in order to stabilize them on their new regimens and to deplete the rats on the low-casein diets of body protein, then they were mated to sibling males. The female rats were kept on the same diets throughout pregnancy and lactation.

Conception was unexpectedly good, even on the lowest protein level, but the offspring were extremely small and would not have lived beyond weaning on diet P1 without additional protein. Although the young of rats fed diet P2 were smaller

than the controls on diet P3, they fared well and were weaned at the normal time. In view of these findings, the lowest level of protein considered to be suitable for representatives of this strain under these experimental conditions lay between those of diets P1 and P2 and the level of 8 per cent was selected. In later experiments with other strains and experimental circumstances, diets P1 and P2 have been found to be ideal. On low-protein diets P1 and P2, the number of pups in a litter had an obvious influence on the size of each individual in that litter. Therefore it was decided, where possible, to standardize the number of rats in each litter in the subsequent experiments.

The main study was designed in the light of the above information from the pilot study and was conducted in two parts using two successive litters from the same females. Sixty-two female rats of the same age and strain as in the pilot study were littermate distributed among three groups and fed synthetic diets P3, P4 and P5 (Table 1). Fifteen females were fed diet P3, fifteen diet P4 and thirty-two diet P5. After 28 days, they were mated with sibling males while maintained on their respective diets, and then continued on the same regimen throughout pregnancy and lactation. Within 3 days after parturition, the litters of the first series were reduced to six, three males and three females where possible, and the pups were weaned when they were 25 days old. One rat was selected at random from each litter at weaning and either transferred to, or in the case of the control animals, continued on the control diet (P3). These subjects were killed at 75 days of age, and the jaws kept for caries examination. The remaining progeny were kept on their respective diets until all their third molars had erupted and were almost in occlusion. Then they were killed and their jaws immediately cleaned of soft tissue and stored in a deep freeze.

Forty-six of the original females were mated again 110 days after the beginning of the experiment. During the whole of this period they had been maintained on the same experimental diets, twenty-four on diet P3 and eleven on each of diets P4 and P5. The remaining sixteen females either had been sacrificed for histological studies or the occasional one had died in the interim between litters. The litters in this second series were not reduced in number, but were divided into two appropriately equal parts at weaning; the animals of one part were continued on the same diet as their mothers, while those of the other part were transferred to one of the other two diets as shown in Table 9. All these rats were killed at 85 days of age, and the heads kept for caries and periodontal disease assessment.

All the animals in both the parental and filial generations were given tap water to drink and their respective diet *ad libitum*. The parents were kept singly in screen-bottomed cages, except when breeding, when they were housed in groups in larger cages with wood shavings for bedding. The offspring were maintained in screen-bottomed cages from weaning until termination of the experimental periods.

RESULTS

The results from the main study can be divided into four parts: (1) effects on growth and development, (2) effects on tooth size and morphology, (3) effects on dental caries incidence, and (4) effects on periodontal disease.

(1) *Effects on growth and development*

The breeding females fed low-protein diet P5 showed a reduced rate of growth within the first 2 weeks on experiment. The average gain in body weight of the females fed diet P5 was 15·4 g in comparison with 26·6 g for the females fed diet P3 with the normal protein level. The rate of conception among the females on diet P5 was unexpectedly good. However, the number of offspring in their litters was appreciably less than for the litters of the control females (Table 2). The pups of the

Table 2. The influence of diets with varying carbohydrate: protein ratios upon the reproductive ability of rats as observed in second series of litters

Maternal diet	No. of females	No. of litters conceived	Av. no. of rats per litter	Av. weight at weaning (g)
P3	11	9	8·5	40·0
P4	11	11	8·8	26·5
P5	24	22	6·2	15·0

low-casein group were much smaller than those of the control females fed diet P3. In addition, their development seemed retarded when judged by the times that first opening of the eyes, development of the external ear and first movement out of the nest were observed. However, despite their small size and sparse hair, the pups from the low-casein females were active and free from infection until they were killed at the end of the experiment. The nipples of the mothers appeared to be dry and withered throughout the suckling period, suggesting that the rate of milk production was low. The quality of the milk may also have been affected.

The pups of mothers fed diet P4 were similar to the control animals in appearance and behaviour, except that they were smaller. Weaning in these experiments was carried out at 25 days instead of the customary 21, because earlier than this the pups of the animals fed diet P5 were too small and dependent to be placed in separate cages.

Those rats which were transferred at weaning from diet P5 to the control diet (P3) showed a marked improvement in growth, but they had not completely recovered from the early influence of the adverse diet by the end of this fairly brief experimental period. At 85 days of age the males were an average of 52 g and the females 31 g lighter than the control animals. If the offspring from low-casein females had been continued on diet P3 for a longer period than 60 days post-weaning, it is possible that, at the observed rate of growth, they would have attained normal adult body weights. Food consumption was not apparently reduced in the adults of the low-casein group, but the offspring fed diet P5 ate less than the control animals, an observation which may have been related to the considerable difference in size.

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The eruption of both the maxillary and mandibular third molar teeth of the offspring fed diet P5 occurred about 5 days later than in the controls. The offspring fed the high-casein:low-sucrose diet (P4) did not show any alteration in the time of eruption of these teeth, even though their average body weight was less than that of the controls.

(2) *Effects on tooth size and morphology*

During the examination of the teeth of the first series of litters, a casual observation was made of a variation in the size and shape of the teeth. In an attempt to measure these differences, twenty rats were selected at random from each of the three groups of offspring and the jaws were photographed by a standard technique in such a way that the occlusal surfaces of the teeth were clearly visible. The enlarged negative images were measured directly along twelve different diameters across the silhouettes of the teeth in such a way that every molar in both jaws was measured in two directions, one antero-posteriorly and the other bucco-lingually (Fig. 1). The measurements were made in arbitrary units and percentage differences calculated (Table 3). The

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TABLE 3. PERCENTAGE REDUCTIONS IN LENGTHS AND WIDTHS OF MOLARS OF OFFSPRING FROM FIRST SERIES OF LITTERS IN LOW-CASEIN (P5) GROUP AS COMPARED TO THOSE FROM CONTROL (P3) GROUP*

Molar	Quadrant	Bucco-lingual width	Mesio-distal length
First	Max.	3·2	3·2
	Mand.	8·1	3·4
Second	Max.	5·3	9·3
	Mand.	7·0	3·7
Third	Max.	10·2	14·9
	Mand.	8·5	15·8

* Since the measurements in this series were made from negative photographic images and expressed in arbitrary rather than absolute units, only the differences are presented.

directions of the lines of measurement were chosen only from a consideration of reproducibility. Low standard deviations within groups confirmed that this was achieved. There were no significant differences between the size of the molar teeth of pups from the high-casein and control groups, but every dimension in the low-casein group was significantly ($P<0·01$) smaller than its counterpart in the other two. The differences were least for the first molars and greatest for the third.

An attempt was made to determine whether these differences in size were due to a fundamental change in the size of the tooth as a whole, or merely due to a difference in enamel thickness. In order to test this, lower first and second molar teeth of rats from both the low-protein and control groups were removed from jaws and ground to predetermined planes in both antero-posterior and bucco-lingual directions. The

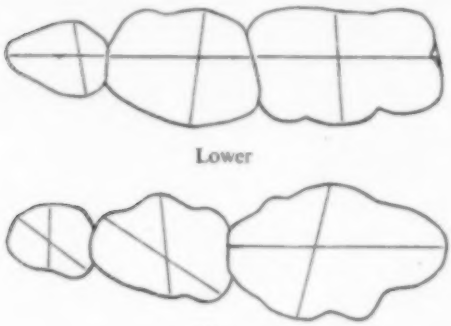

FIG. 1. Diagrammatic outlines of the occlusal surfaces of rat molars. The two straight lines drawn across each molar represent the antero-posterior and bucco-lingual dimensions that were measured on negative photographic images. The differences in these dimensions between rats from protein-deficient females and rats from control females are presented in Table 3.

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ground surfaces were coloured with van Gieson's stain to delineate clearly the amelo-dentinal junctions, and viewed under a travelling microscope. Measurements were made across the surfaces to an accuracy of 0·0001 in. A baseline measurement was taken first from the amelocemental junction on one side to that on the other. All other measurements were made parallel to this baseline, both across the outer dimensions of the teeth and from the amelodentinal junction on one side to that on the other. The difference between these two measurements represents the thickness of the enamel (Fig. 2). The results are presented in Table 4. It can be seen that these more precise measurements confirm the previous results showing differences in size of the teeth. In addition, all the measurements made across the teeth from the amelodentinal junction of one side to that of the other were consistent in showing that the teeth of the low-casein group were basically smaller than those of the control animals. No such differences between these two groups were demonstrated in enamel thicknesses.

Not only differences in size but also differences in shape of teeth were noticed. Some of the subsidiary cusps of the teeth of the low-casein: high-sucrose group (P5) were affected, particularly on the third molars. The teeth of rats in the second series of litters were examined to determine the extent of these differences. Some cusps were obviously reduced in size and others were missing altogether. This was particularly noticeable with regard to the small antero-buccal cusp of the lower third molar tooth. As it is difficult to measure accurately differences in size of cusps, and as this observation is, therefore, rather subjective in nature, only those cusps which were absent altogether are included in the data presented in Table 5. Cusps were considered missing if no evidence could be found of their presence when the surfaces of the teeth were viewed under a dissecting microscope at a magnification of twenty diameters. Although these cusps tend to be missing occasionally in the

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TABLE 4. THE INFLUENCE OF THE MATERNAL DIET UPON THE MEASUREMENTS ACROSS SURFACES OF TEETH IN SECOND SERIES OF LITTERS (IN INCHES)

	Maternal diet	Mandibular first molar						Mandibular second molar					
		Bucco-lingual dimensions			Antero-posterior dimensions			Bucco-lingual dimensions			Antero-posterior dimensions		
		No. of teeth	Average	Diff. P	No. of teeth	Average	Diff. P	No. of teeth	Average	Diff. P	No. of teeth	Average	Diff. P
Total width of teeth	P3	10	0·0680	0·0030 <0·02	9	0·1166	0·0039 <0·02	10	0·0758	0·0070 <0·001	12	0·0793	0·0049 <0·001
	P5	9	0·0650		11	0·1127		10	0·0688		11	0·0744	
Width between amelo-dentinal junctions	P3	10	0·0614	0·0035 <0·01	9	0·1097	0·0038 <0·02	10	0·0670	0·0074 <0·001	12	0·0709	0·0048 <0·001
	P5	9	0·0579		11	0·1059		10	0·0596		11	0·0661	
Enamel thicknesses	P3	10	0·0066	0·0005 >0·05	9	0·0069	0·0002 >0·5	10	0·0088	0·0004 >0·1	12	0·0084	0·0001 >0·5
	P5	9	0·0071		11	0·0067		10	0·0092		11	0·0083	

FIG. 2. Diagram of a ground section of a rat molar in the sagittal antero-posterior plane. The lines across the section are drawn to represent the places where measurements were made to determine whether reductions in size were due to enamel thickness or to a reduction in the distance between the outer borders of the dentine. The data based on these measurements are presented in Table 4.

offspring from the P3 and P4 females, they were missing in a high percentage of the rats born to P5 females. In addition, the right maxillary third molar was congenitally absent in one rat born to a female on the P5 diet. It can also be seen from Table 5 that the cusp pattern of the third molar appears to be fixed before 25 days of age, for changes in diet at this stage had no apparent effect upon the presence of this cusp.

TABLE 5. RELATION OF DEVELOPMENTAL DIET TO THE CUSPAL PATTERN OF THIRD MOLAR TEETH

Maternal diet	Post-weaning diet	Total no. of rats	No. of rats with missing cusps on one or more third molars
P3	P3	34	2
P3	P5	31	1
P4	P4	33	3
P4	P3	34	5
P5	P5	58	40
P5	P3	58	41

(3) *Effects on dental caries incidence*

The results from the first series of litters are presented in Table 6. All the rats received the control diet (P3) from the time they were weaned at 25 days until they were killed 50 days later. The dental caries incidence was assessed as in previous studies ((SHAW *et al.*, 1944) by determining the number of molars affected, the number of carious lesions and the extent of carious lesions.

Quite clearly, those rats under the nutritional influence of the high-casein:low-sucrose diet (P4) up to 25 days of age have a significantly lower dental caries incidence

TABLE 6. THE RELATIONSHIP OF THE MATERNAL DIET TO THE DENTAL CARIES INCIDENCE IN REPRESENTATIVES FROM FIRST SERIES OF LITTERS

Maternal diet	No. of rats	No. of carious molars			No. of carious lesions			Extent of carious lesions		
		Av.	Diff.	*P*	Av.	Diff.	*P*	Av.	Diff.	*P*
P4	13	1·2	2·9	<0·01	1·8	4·0	<0·02	2·3+	6·8+	<0·02
P3	13	4·1	2·6	<0·01	5·8	5·5	<0·01	9·1+	21·5+	<0·001
P5	23	6·7			11·3			30·6+		

than the control group (P3) ($P<0·02$), and those under the influence of the low-casein:high-sucrose diet (P5) up to 25 days of age have a significantly higher dental caries incidence than the control group ($P<0·01$).

As the molar teeth erupt at slightly different times, and, except for the third molars, before 25 days of age, individual molar teeth were compared to see whether the differences in caries experience could be explained by a short initial exposure to the different developmental diets. The results are shown in Table 7. The magnitude of the differences between the teeth of pups from groups P3 and P4 was similar for the lower first and second molar teeth. When the teeth of pups from groups P3 and P5 were compared, it could be seen that the differences were more marked in the lower first molar teeth than in the lower second, but those in the latter were still apparent ($P<0·05$). Caries incidence in the lower third molars of all groups was so low that no comparisons could be made. This was partly because of the usual high resistance of the third molars in the rat to dental caries, and partly because of the very short interval that the third molars in this experiment were exposed to the oral cavity. In the case of the pups of the P5 group, these teeth would only have been erupted about 35 days.

The study on the second series of litters made it possible to compare both the developmental and environmental influences of these various diets. The details are presented in Table 8. Those animals whose mothers were fed diet P4 and who were continued on this diet for the full experimental period remained caries-free. However, their littermates, who were transferred to diet P3 at weaning, had appreciable amounts of tooth decay. The rats whose mothers were fed diet P3 and who were continued on this diet had significantly more carious lesions than either of the previous groups ($P<0·01$). Thus the difference in dental caries activity between the rats with the complete history on diet P4 and those wholly on diet P3 appears to be due partly to a developmental and partly to an environmental influence. There is also a marked difference between the caries incidence of the rats with the complete P3 history and those receiving the low-casein:high-sucrose diet (P5) throughout both periods ($P<0·01$). In this case the increased caries activity among the pups from the P5 females is apparently due entirely to a developmental influence, since the feeding of either diet P3 or P5 in the post-weaning period did not cause a difference in the caries incidence.

TABLE 7. THE RELATIONSHIP OF THE MATERNAL DIET TO THE DENTAL CARIES INCIDENCE IN INDIVIDUAL MANDIBULAR MOLARS—FIRST SERIES OF LITTERS

Maternal diet	No. of rats	No. of carious molars				No. of carious lesions				Extent of carious lesions			
		First molar			Second molar					First molar			Second molar		
		Av.	Diff.	P	Av.	Diff.	P	Av.	Diff.	P	Av.	Diff.	P	Av.	Diff.	P
P4	13	0·5	1·4	<0·001	0·4	1·5	<0·01	1·1	2·3	<0·01	0·5	1·7	<0·02	1·5+	3·6+	<0·05	0·5+	3·2+	<0·01
P3	13	1·9	1·4	<0·001	1·9	1·1	<0·05	3·4	3·3	<0·001	2·2	2·3	<0·01	5·1+	14·4+	<0·001	3·7+	7·1+	<0·01
P5	23	3·3			3·0			6·7			4·5			19·5+			10·8+		

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TABLE 8. THE INFLUENCE OF THE MATERNAL AND POST-WEANING DIETS UPON THE DENTAL CARIES INCIDENCE IN SECOND SERIES OF LITTERS

Group	No. of rats	Diets		No. of carious molars			No. of carious lesions			Extent of carious lesions		
		Pre-weaning	Post-weaning	Av.	Diff.	*P*	Av.	Diff.	*P*	Av.	Diff.	*P*
1	33	P4	P4	0			0			0		
					2·5	<0·001		3·8	<0·001		9·5+	<0·01
2	34	P4	P3	2·5			3·8			9·5+		
					2·1	<0·01		3·6	<0·01		14·1+	<0·001
3	34	P3	P3	4·6			7·4			23·6+		
					0·2	>0·5		0·1	>0·5		1·2+	>0·5
4	31	P3	P5	4·4	2·2	<0·001	7·5	5·8	<0·001	22·4+	25·0+	<0·001
5	58	P5	P3	6·4	1·8	<0·01	12·6	5·2	<0·01	42·0+	18·4+	<0·001
					0·2	>0·5		0·7	>0·5		5·4+	>0·1
6	58	P5	P5	6·6			13·3			47·4+		

(4) *Effects on periodontal disease*

During the examination of the jaws of the first series of litters, both gingival recession and alveolar bone resorption were noticed. Because of this, all the jaws of the second series of litters were carefully examined for these two conditions and scored by the method of SHAW and GUPTA (1956) previously applied to periodontal disease in rice rats. The results of both soft and hard tissue examinations are given in Table 9.

When the results for the rats fed diet P4 all their lives are compared with those of the control group, it can be seen that the former show both marked gingival recession and alveolar bone resorption. By comparing the scores of both these groups with those of the rats transferred at weaning from diet P4 to diet P3, it is apparent that the adverse influence of diet P4 on the integrity of both the gingival tissue and the alveolar bone is evident both prior to and following the eruption of the teeth. Although the effect is slightly more marked in the latter period, both are well established ($P<0·01$). When compared with the results on the high-casein regimens, low-casein diets had little effect on this periodontal syndrome. None of the groups receiving diet P5 showed significant differences in alveolar bone resorption when compared with the control group. However, there were small effects on the amount of gingival recession. When the rats fed diet P5 throughout their whole lives are compared with the control group, or either of the groups fed the low-casein diet for part of their existence, it will be seen that they have a significantly greater amount of gingival recession ($P<0·05$). However, it would seem that feeding diet P5 for only part of their life cycle made little difference.

TABLE 9. THE INFLUENCE OF THE MATERNAL AND POST-WEANING DIETS UPON THE PERIODONTAL DISEASE SCORES IN SECOND SERIES OF LITTERS

Group	No. of rats	Diets		A*			B*			C*			D*			E*			F*		
		Pre-weaning	Post-weaning	Av.	Diff.	*P*	Av.	Diff.	*P*	Av.	Diff.	*P*	Av.	Diff.	*P*	Av.	Diff.	*P*	Av.	Diff.	*P*
1	33	P4	P4	7·3	4·4	<0·001	18·2	10·9	<0·001	16·0	10·8	<0·001	10·0	3·7	<0·001	27·6	12·3	<0·001	25·6	14·3	<0·001
2	34	P4	P3	2·9	2·0	<0·01	7·3	6·0	<0·01	5·2	4·5	<0·01	6·3	2·2	<0·01	15·3	7·1	<0·01	11·3	6·3	<0·001
3	34	P3	P3	0·9	0·6	=0·1	1·3	0·8	<0·1	0·7	0·4	=0·5	4·1	0·2	>0·5	8·2	2·7	<0·1	5·0	3·8	>0·1
4	31	P3	P5	0·3	1·9	<0·01	0·5	5·6	<0·01	0·3	3·9	<0·01	3·0			6·2			3·6		
5	58	P5	P3	0·7	0·2	>0·5	1·1	0·2	>0·5	0·6	0·1	>0·5	4·4			10·1			6·8		
6	58	P5	P5	2·2	1·5	<0·01	6·1	5·0	<0·02	4·2	3·6	<0·05	4·3			10·9			8·8		

* A = No. of molars with contiguous soft tissue areas affected by periodontal syndrome.
B = No. of soft tissue areas affected by periodontal syndrome.
C = Extent of soft tissue damage.
D = No. of molars with contiguous areas of calcified tissue affected by periodontal syndrome.
E = No. of calcified tissue areas affected by periodontal syndrome.
F = Extent of calcified tissue damage.

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DISCUSSION

The genetic constitution of an animal provides a template that, under optimal systemic conditions during development, determines with considerable precision the shape, size and caries-susceptibility of individual teeth. When adverse conditions prevail during tooth development, reproduction of the exact specifications prescribed by the template may not be possible. Good examples of some of the gross aspects of this interrelationship have been described by PAYNTER and GRAINGER (1956), who observed that a small fluoride supplement, or a vitamin A deficiency or the use of a low-calcium:high-phosphorus ration during tooth development in the rat resulted in smaller molars. In addition, the depth and breadth of the sulci of the molars were altered, particularly as a result of prolonged maintenance on the diet with the moderately low-calcium:high-phosphorus content (ratio 1:3).

In the present study, it has been possible to provide more specific information about the relationship between the systemic conditions prevailing during tooth development and the genetic constitution of the rat. The offspring of the females fed the low-protein:high-sucrose diet (P5) grew more slowly and their teeth erupted later than the controls; they also had significantly smaller molars. This change in size was due to a decrease in the distance between the outer borders of the dentine rather than to a reduction in enamel thickness. In addition, many of these rats had missing cusps on the third molars and in one the absence of a maxillary third molar was also noted. The latter event has never before been observed in well over 10,000 representatives of this strain. These changes in the size and morphology of the molars must have been the result of a systemic influence operating early in tooth development, because the size and shape of the amelodentinal junction is determined before any calcification of enamel or dentine occurs.

The rats of the Harvard strain used in this experiment are highly susceptible to dental caries under the conditions prevailing for the control subjects; however, the experimental circumstances imposed on the offspring of the females fed diet P5 resulted in a highly significant increase in susceptibility to dental caries under standardized post-developmental conditions. Indeed, the caries-susceptibility of these rats was greater than in any previous group of this strain subjected to a developmental stress. These differences in caries-susceptibility are related definitively to the developmental period of the teeth and to the diets fed to the mothers throughout pregnancy and lactation. In addition, there were post-eruptive effects of the various diets on caries activity that can be explained on popularly held theories. As would be expected, the rats fed the high-protein:low-sucrose diet (P4) post-eruptively developed a low level of caries activity when compared with those fed diets containing lower casein and higher sucrose levels (P3 and P5). The similar post-eruptive caries-producing influences of diets P3 and P5 were not unexpected, even though they contained as different levels of sucrose as 67 and 83 per cent, respectively. In a caries-susceptible population of rats, major reductions in soluble carbohydrate content below 67 per cent are necessary in order to achieve a significant reduction in caries activity.

The strain of rats used in this study has never exhibited any appreciable evidence of a susceptibility to periodontal disease, and indeed, little evidence was observed

among the offspring of the control females. However, in the offspring of the females fed the high-casein: low-sucrose diet (P4), a definite tendency toward a breakdown of the supporting structures was observed; the offspring of the females on diet P5 also showed some evidence of the periodontal syndrome but at appreciably lower levels than the offspring of the females fed diet P4. The severity of the periodontal lesions was influenced by these diets both prior to and following the eruption of the teeth.

These periodontal results were both unexpected and difficult to explain, especially since both developmental and post-developmental influences were implicated. In general, the albino rat is considered to be unsuitable for studies of this disease because of its relative resistance. None of the subjects in any group in this study exhibited a comparable severity of periodontal disease to that obtained in the rice rat (Shaw and Gupta, 1956). The Harvard susceptible strain has been selectively inbred for caries-susceptibility. It was of interest to note that, whereas the amount of periodontal disease showed considerable variation between subjects within groups, the caries scores were remarkably similar. This observation is a further demonstration of the value of genetic uniformity within experimental subjects. However, the same observation indicates that, although the strain is approaching homogeneity with respect to caries-susceptibility, comparable homogeneity has not been achieved with respect to other characteristics, such as susceptibility to a periodontal syndrome.

One of the most surprising aspects of this study was the excellent breeding response of the low-protein group. In anticipation of poor reproduction, this group had been heavily weighted in the number of females in the original experimental plan. However, an excellent rate of conception was observed in the protein-depleted females, culminating in the birth of viable young with a good number of pups per litter. After birth, the females in the low-protein group were less well able to provide for their offspring, probably due to the lack of adequate milk, or due to milk of inferior quality. Hence their offspring were late in all stages of development. This lag in development had to be considered when the time came to kill the animals. Most of the rats from the first series of litters were to be used to provide tooth substance for chemical investigations, and in view of that it seemed more appropriate to kill those in the various groups at the same developmental stage rather than the same chronological age (Stack and Holloway, 1959). For this reason they were killed on eruption of the third molar teeth. This meant that the average age of the animals of the P5 group at death had to be 5 days more than that of the other two in order to have a comparable state of development. The chemical analyses of these samples have not been completed and the results will be reported later.

One of the greatest difficulties in assessing the specific nature of the pre-eruptive influences involved in a study of this nature results from the fact that the molar teeth of rats develop during foetal and early post-natal life. Thus when an attempt is made to influence these teeth during their development, the imposed nutritional regimen may be radically modified, first by the various maternal systemic mechanisms that operate to moderate the influence, second by the placental barrier, and third by the mammary glands. Coprophagy and refection are additional influences modifying the nutritional value of diets.

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On the basis of this study, the influences upon the size, morphology and caries-susceptibility of teeth and upon the periodontium of the offspring cannot be equated to a simple alteration in protein intake. However, it can be concluded that the nutritional influences imposed in this experiment upon the maternal organism by the isocaloric substitution of sucrose by casein have resulted in the various changes in the offspring described. Among the young of the low-casein:high-sucrose (P5) females, inanition undoubtedly played some part, as evidenced by the poor rate of growth, and by suggestions of a low rate of lactation and possibly of milk of inferior quality. Many studies involving protein deficiencies can be controlled accurately by paired feeding techniques in order to counteract any effects that inanition, etc., may produce. However, in this study where the experimental subjects must be influenced *in utero* and during the suckling period, the application of the paired feeding technique to the mothers would be irrelevant. In the offspring of the high-casein:low-sucrose (P4) females, alterations in the acid-base relationships as a result of the utilization of large amounts of protein as an energy source may have influenced the development of the oral structures. As the casein content of the diets was varied, its appreciable phosphate level caused a small but definite variation in the phosphate level of the three rations. Although the amount of phosphate introduced by the additional casein was less than that expected to cause a reduction in caries activity, this variant is being explored currently in another experiment of comparable design.

Thus it can be seen that the varied effects upon the oral structures described in this work cannot be associated with one specific nutritional factor. At present, they must be considered to be the resultants of the entire experimental circumstance until such time as more specific isolation of the various influences can be achieved and evaluated experimentally.

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DENTAL PULP FLUID—I

RELATIONSHIP BETWEEN DENTAL PULP FLUID AND BLOOD PLASMA IN PROTEIN, GLUCOSE AND INORGANIC ELEMENT CONTENT

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Abstract—When a hole is drilled in the dog's tooth with a Cavitron or an Airdent, the pulp chamber can be reached or approximated without injury to the pulpal blood vessels. A capillary tube is inserted in the hole and cemented in place, whereupon a clear, colourless fluid begins to rise in the tube. Over a period of several hours a sufficient quantity of the fluid, designated as dental pulp fluid, can be collected for chemical analysis. The protein content of the dental pulp fluid is about one-fifth that of the blood plasma, whereas the glucose concentration is in equilibrium with the concentration in arterial blood plasma. Spectrographic analysis of dental pulp fluid and blood plasma showed that magnesium, iron, calcium, phosphorus, copper and sodium are present in both fluids.

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INTRODUCTION

BODECKER (1911) showed from histological preparations the presence of organic material in the enamel of teeth and its continuity with that in the dentine. VON BEUST (1912) reported experiments to demonstrate that "the lines, or canals, seen by former observers are channels through which plasma can reach all parts, generally speaking, of the enamel." In one experiment he immersed the tip of the root in a strong alcoholic solution of fuchsin; in another he injected a staining solution into the pulp. In both instances the staining material passed through the dentine into the enamel.

The objection was raised that these experiments were done on extracted teeth and consequently did not prove that there is circulation of fluid in the tooth *in vivo*. This question was settled by the experiments of FISH (1926, 1927a,b). Solutions of stains such as trypan blue, and India ink, were injected subcutaneously into cats and dogs, the animals sacrificed at various intervals after injection and the teeth sectioned for examination. In as short a time as half an hour, a faint colour was seen in the dentine, which became more intense with longer intervals after injection. Penetration of the dye into the enamel was also observed. FISH concluded that the tubules may be looked upon as lymph channels whereby nutrient materials, oxygen and immune bodies are transported to the living dentine and the products of its catabolism carried away.

BODECKER (1923, 1929) supplied further evidence in support of the idea that fluid, which he also designated as dental lymph, circulates through the dentine and enamel

of the tooth. In later experiments, BODECKER and LEFKOWITZ (1937) cut a small cavity in the tooth and packed a dry dye into its lower portion. They then sealed the cavity and later extracted the tooth, whereupon they found the dye in various areas within the tooth. Obviously, there must have been, as BODECKER had previously contended, fluid in the tooth substance to dissolve the dye, and also circulation of the fluid within the tooth to transport the dye to other areas. Later, LEFKOWITZ (1943) found that dye inserted into the pulp chamber of the dog's tooth penetrated the dentine within 13 min and extended approximately the length of the tubules. In about 17 min it had reached the periphery of the tubules in the cervical area. More recent studies with radioisotopes have confirmed the conclusions of these earlier investigators with respect to passage of body fluids through the tooth (VOLKER and SOGNNAES, 1940; ARMSTRONG and BARNUM, 1948; SOGNNAES, SHAW and BOGOROCH, 1955).

VON KREUDENSTEIN and STUEBEN (VON KREUDENSTEIN, 1955; VON KREUDENSTEIN and STUEBEN, 1955, 1956; STUEBEN and VON KREUDENSTEIN, 1956) employed the method of cutting off the crown above the pulp cavity by means of a diamond abrasive disc and heating the removed portion of the crown (VON KREUDENSTEIN and STUEBEN, 1955). Numerous droplets appeared on the ground surface of the dentine and coalesced into larger droplets. This fluid was referred to by VON KREUDENSTEIN and STUEBEN as "dentinliquor" or dentinal fluid. They have made various chemical analyses of the fluid which was collected by elution with distilled water or physiological saline. The present report is concerned with the quantitative relationship between the protein and glucose content of the dental pulp fluid and the blood plasma from the same animal and the qualitative relationship between the inorganic elements in these two fluids.

The presence of capillaries in the pulp chamber suggested to us the likelihood of an exchange of fluid in this locality similar to that which occurs in the soft tissues. If the fluid could be obtained in sufficient quantities, chemical analyses of the fluid and of the blood plasma obtained simultaneously from the same animal, should throw light on the mechanism whereby the fluid was produced.

MATERIAL AND METHOD

The pulp chamber in dogs' teeth was reached by cautiously drilling a hole with a Cavitron (Cavitron Corporation, New York City) or Airdent (S. S. White Manufacturing Company, Philadelphia). This was accomplished without damage to the blood vessels.

A droplet of clear fluid could usually be seen to emerge from the pulp chamber. A capillary glass tube was inserted into the hole and cemented in place. After several hours, a clear, colourless liquid rose in the tube and, in most instances, in an amount sufficient for micro-chemical analyses. In the early stages of the experiment, we learned that fluid could be collected from the incisors, premolars and molars, and that analysis of the fluid from these different teeth gave the same results. The amount of fluid that can be collected varies from one experiment to another; in fact, in some experiments we have been unable to collect any fluid. The reason for these variations is at present under investigation. Fig. 1 shows a longitudinal section of a dog's canine

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tooth to show the depth to which the hole was drilled. Fluid was obtained from this tooth.

The fluid obtained by our procedure will be referred to as dental pulp fluid. This we believe to be a more appropriate designation than "dental lymph" inasmuch as the fluid presumably is not derived from lymph vessels. Each sample of fluid was routinely examined microscopically for erythrocytes and leucocytes. We have found almost every collection to be free of both red and white blood cells, and in the very few instances where these were present, the samples were discarded. The absence of erythrocytes was taken to indicate that the blood vessels had not been ruptured. Obviously, the fluid we obtained was a normal physiological fluid and not an inflammatory exudate, for if it were an exudate, leucocytes and most likely erythrocytes also would have been present and the protein content would have been at a considerably higher level.

Protein determinations were made on twenty-five collections of pulp fluid and blood plasma obtained at different times on fourteen dogs and glucose analyses on twelve samples of pulp fluid and arterial and venous blood plasma from five dogs on different days. Nitrogen determinations were made by a modified Kjeldahl micro method on 0·01–0·02 ml blood plasma or pulp fluid which was digested with sulphuric acid and potassium sulphate and selenium. The ammonia was released by sodium hydroxide and steam distilled into 2 or 4% boric acid, using the micro Kjeldahl distillation apparatus. Titration with standard acid was carried to an end point that matched the blank. Protein was calculated as $N \times 6{\cdot}25$. In other experiments glucose determinations were made on the dental pulp fluid and on the plasma of arterial and venous blood drawn simultaneously from the femoral vein and femoral artery. The micro version of the Nelson–Somogyi method (NATELSON, 1957) was used for glucose analysis on 0·01 or 0·02 ml plasma or pulp fluid, which was washed into 0·5 ml distilled water. Protein was precipitated with barium hydroxide and zinc sulphate, which proved to give better checks than precipitation with tungstic acid. A 1 ml aliquot of the supernatant fluid was heated with copper reagent for 10 min. It was then cooled and mixed with 1 ml arsenomolybdate reagent. The standards and unknown were read at 540 λ or with a 54 filter. The presence of inorganic elements in blood plasma and dental pulp fluid was determined spectrographically by placing 0·02 ml of either plasma or pulp fluid on a carbon electrode which was allowed to dry and then ignited by an arc in a Bausch and Lomb spectrograph. Comparative observations were made of the spectral lines on the spectrographic plate.

RESULTS

The average protein concentration in the pulp fluid was 1·2 g per cent as compared with 6·6 g per cent in venous blood plasma. The individual data are presented in Table 1 so as to allow comparison of the data on the blood plasma and pulp fluid on different animals and on collections from the same animal on different days. An interval of several days elapsed whenever more than one experiment was done on the same animal. The successive collections of fluid on different days were obtained from teeth that had not been drilled in a previous experiment.

TABLE 1. PROTEIN (N × 6·25) CONTENT OF DENTAL PULP FLUID AND BLOOD PLASMA

Dog	Blood plasma (g %)	Dental pulp fluid (g %)	Dog	Blood plasma (g %)	Dental pulp fluid (g %)
1	6·6	1·1	9	6·9	1·2
2	6·4	1·1	9	7·0	1·3
2	6·7	1·1	10	6·7	1·2
3	7·0	1·2	10	6·6	1·2
3	7·0	1·1	10	6·7	1·1
3	6·9	1·1	11	6·6	1·0
4	6·7	1·3	11	6·6	1·2
5	6·9	1·3	12	6·3	1·2
5	7·0	1·2	12	6·4	1·2
6	6·5	1·2	13	6·4	1·2
7	6·3	1·2	13	6·4	1·3
7	6·3	1·0	14	6·3	1·2
8	7·0	1·3	14	6·4	1·2
8	6·8	1·2	14	6·7	1·1
			Average	6·6	1·2

Samples from the same dog were obtained at intervals of several days.

TABLE 2. GLUCOSE CONTENT OF DENTAL PULP FLUID AND OF VENOUS AND ARTERIAL BLOOD PLASMA

Dog	Venous blood plasma (mg %)	Arterial blood plasma (mg %)	Dental pulp fluid (mg %)
15	73	86	85
16	75	80	79
16	80	84	80
16	76	82	80
17	70	76	75
17	72	80	82
18	81	91	93
18	80	90	90
18	78	85	86
19	78	89	90
19	82	91	92
19	81	89	90
Average	77	85	85

Samples from the same dog were obtained at intervals of several days.

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As may be seen from the data in Table 2, the glucose concentration in the dental pulp fluid was higher than in venous blood plasma and at the same level as in arterial plasma.

In those experiments in which pulp fluid, arterial and venous blood plasma were examined spectrographically, the plates showed the same magnesium, iron, calcium, phosphorus, copper and sodium lines for the three fluids.

DISCUSSION

The results of these experiments indicate that in the pulp chamber the situation resembles that in the soft tissues where certain constituents of the blood pass through the capillary walls into the intercellular spaces and eventually into the lymphatics. It is impossible to draw a strict comparison between the dental pulp fluid and the lymph inasmuch as the lymph formed in different parts of the body varies in its composition. However, these two fluids appear to be analogous. The protein content is considerably less than in the blood plasma, presumably because the capillary epithelium is, relatively speaking, only slightly permeable to protein, and in particular to the larger globulin molecule. Glucose, on the other hand, passes freely through the capillaries in the pulp chamber just as it does elsewhere in the body. It is evident from the spectrographic analyses that the inorganic elements in the plasma pass through the capillary walls, although we are not in a position at the present time to say whether there is any difference in the concentration of these elements in the pulp fluid and in the blood plasma.

The composition of the dental pulp fluid in these experiments is different from that of the "dentinliquor" obtained by VON KREUDENSTEIN and STUEBEN. The protein content of the latter was 150 mg per cent (cited by VON KREUDENSTEIN, 1958) as compared with an average concentration of 1200 mg per cent which we obtained in the dental pulp fluid. Similarly in the experiments of STUEBEN and VON KREUDENSTEIN (1956), the "dentinliquor" was found to contain about 45 mg per cent reducing substances or only about half that normally found in the blood, whereas the concentration of reducing substances in the dental pulp fluid in our experiments, designated in Table 2 as glucose, were in equilibrium with those in the arterial blood plasma.

In the procedure described by VON KREUDENSTEIN (1955) the "dentinliquor" was forced out of the tooth by rapidly raising the temperature from 22 to 90°C. We are therefore confronted with several questions. Does rapid heating cause a coagulation of some of the protein material in the dentine thereby trapping some reducing substances and resulting in a difference in concentration between the "dentinliquor" (VON KREUDENSTEIN) and our dental pulp fluid? Is there some resistance or obstruction to the passage of certain protein molecules through the dentine? Is there a loss of protein and of glucose to the dentinal substance as the dental pulp fluid passes through the tooth? These questions cannot be answered at the present time.

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FIG. 1. Longitudinal section of dog's premolar in which the hole was drilled.

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THE APPLICABILITY OF KNUTSON'S INDEX OF PREVALENCE OF DENTAL CARIES TO DATA FOR AUSTRALIAN, FORMOSAN AND INDONESIAN CHILDREN

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Abstract—Knutson's index for prevalence of dental caries and the conversion table derived from it have been applied to data on dental caries prevalence among Australian, Indonesian and Formosan children. Although other equations may be derived which will more precisely fit these observations, the Knutson equation $(97-y)=97\ (0{\cdot}524)^x$ and the corresponding conversion table are satisfactory for public health purposes.

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INTRODUCTION

EVIDENCE was presented by KNUTSON in 1944 showing a relationship between two measures of the prevalence of dental caries, namely the proportion of children by age with one or more carious (DMF) teeth and the average number of carious (DMF) teeth per child. Data were presented for the children in yearly age groups and an equation was derived which describes this association and which enables the average number of DMF teeth per child to be calculated from a knowledge of the proportion of the group with one or more DMF teeth. This equation, of the catalytic type, is expressed as $K-y=KB^x$ where K and B are constants, y is the percentage of the group with one or more DMF teeth and x is the average DMF teeth per child for the group. The values derived by KNUTSON for the data in his paper were $K=97$ and $B=0{\cdot}524$ and the equation using these values was shown to be valid for data from the U.S.A. up to certain values for the proportion of children with one or more DMF teeth. The size of the groups examined and the range of values for DMF teeth per child to be expected through sampling error were discussed, and the suggestion made that observations should be limited to ages below those at which 90 per cent were likely to be found with one or more DMF teeth. Estimates of caries prevalence at other ages could then be obtained by linear interpolation and extrapolation.

KNUTSON has pointed out the rapidity with which the prevalence of dental caries in children can be assessed by a simple examination to determine merely the presence or absence of caries, followed by the use of a conversion table to ascertain the average DMF per child, by age. He also has emphasized the added advantages of its low

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cost and its adequacy for surveys in connexion with public dental health programmes, where the main purposes are usually to indicate the magnitude of the task confronting a treatment service or the effectiveness in a community of caries control measures.

Furthermore, as there is a direct relationship between the number of DMF teeth per child and the number of DMF tooth surfaces per child (BARNARD, 1956; KNUTSON, 1958), the simplified survey enables a satisfactory estimate to be made firstly of the mean DMF teeth per child and secondly of the mean DMF tooth surfaces per child

During the discussions at the Second Seminar on Dental Health held at Adelaide, South Australia, sponsored by the Western Pacific Regional Office of the World Health Organization, the validity of this relationship between percentage with one or more DMF teeth and average DMF teeth per child was questioned for countries other than the U.S.A., particularly for those of South-East Asia and the Western Pacific Regions represented at the Seminar. Discussion was centred firstly on its general application and secondly on the use of the conversion table based on Knutson's equation (Table 1) to derive satisfactory working estimates of caries prevalence in terms of average DMF teeth per child. Because tests of the validity of the conversion

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TABLE 1. CONVERSION TABLES DERIVED FROM EQUATIONS OF THE FORM $K-y=K(B)^x$

Percentage with one or more DMF teeth	Average DMF per child		
	Knutson's equation $97-y=97\ (0{\cdot}524)^x$	$100-y=100\ (0{\cdot}521)^x$	$93-y=93\ (0{\cdot}54)^x$
(y)	(x)	(x)	(x)
99		7·05	
98		6·00	
97		5·34	
96	7·08	4·94	
95	6·01	4·59	
90	4·07	3·52	
85	3·23	2·91	3·80
80	2·69	2·50	3·20
75	2·30	2·12	2·68
70	1·98	1·85	2·30
65	1·72	1·61	2·00
60	1·49	1·41	1·75
55	1·30	1·22	1·56
50	1·12	1·06	1·38
45	0·96	0·85	1·19
40	0·82	0·78	1·05
35	0·69	0·66	0·90
30	0·57	0·55	0·77
25	0·46	0·43	0·65
20	0·36	0·34	0·52
15	0·26	0·25	0·40

table had not been made on data for countries other than the U.S.A. the following recommendation appears in the report of the Seminar (WORLD HEALTH ORGANIZATION, 1959a, p. 27), "it is recommended that studies be made of the applicability of the DMF conversion table in determining the 'working estimate' to other countries, particularly those of South-East Asia and the Western Pacific."

An attempt has been made in this report to assess the applicability of the conversion table based on the equation $97-y=97\,(0{\cdot}524)^x$ to Australian, Formosan and Indonesian data.

DATA

The data for Australian school children were obtained from a survey of a random sample of children in urban and rural communities in New South Wales by BARNARD (1956). Data for Formosan school children were taken from a survey in that country (WORLD HEALTH ORGANIZATION, 1959b). The figures for Indonesian children are those presented by SOERIA SOEMANTRI (1959) in his report on dental health services in Indonesia to the second Seminar on Dental Health held in Adelaide (Table 2).

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TABLE 2. DENTAL CARIES PREVALENCE AMONG CHILDREN IN AUSTRALIA, FORMOSA, INDONESIA AND THE U.S.A.

	Age last birthday (years)										
	6	7	8	9	10	11	12	13	14	15	16
Australia (*New South Wales*) (BARNARD, 1956)											
Number of children	710	744	745	731	689	717	667	696	660	428	
Percentage with one or more DMF teeth	41·3	76·5	89·5	95·9	95·4	97·5	99·0	99·6	99·4	100	
Average DMF per child	0·99	2·31	3·22	4·44	5·28	6·98	9·32	10·70	12·78	13·91	
Formosa (WORLD HEALTH ORGANIZATION, 1959b)											
Number of children			284		475	853					
Percentage with one or more DMF teeth			67·0		54·4	55·4					
Average DMF per child			1·38		1·22	1·44					
Indonesia (*Bandung area*) (SOERIA SOEMANTRI, 1959)											
Number of children	1237	2485	2435	2689	2339	2201	2550	2101	1468	541	149
Percentage with one or more DMF teeth	25·8	41·3	51·9	62·3	65·1	67·4	71·4	78·4	79·9	82·8	85·2
Average DMF per child	0·54	0·96	1·27	1·64	1·91	2·21	2·58	3·14	3·51	3·35	3·80
U.S.A. (*Hagerstown*) (KNUTSON, 1944)											
Number of children	327	403	487	493	529	531	596	565	695	651	
Percentage with one or more DMF teeth	15·9	36·2	53·4	70·6	78·3	81·9	87·8	91·2	94·8	95·4	
Average DMF per child	0·29	0·73	1·20	2·02	2·51	2·84	3·66	4·55	5·62	6·64	

RESULTS

Australian children

When Barnard's data for Australian children were plotted in the form of percentage with one or more DMF teeth against average DMF teeth per child a curve similar to Knutson's curve for Hagerstown was obtained (Fig. 1), except that it reached a higher percentage closely approximating 100 per cent. In fact the proportion of

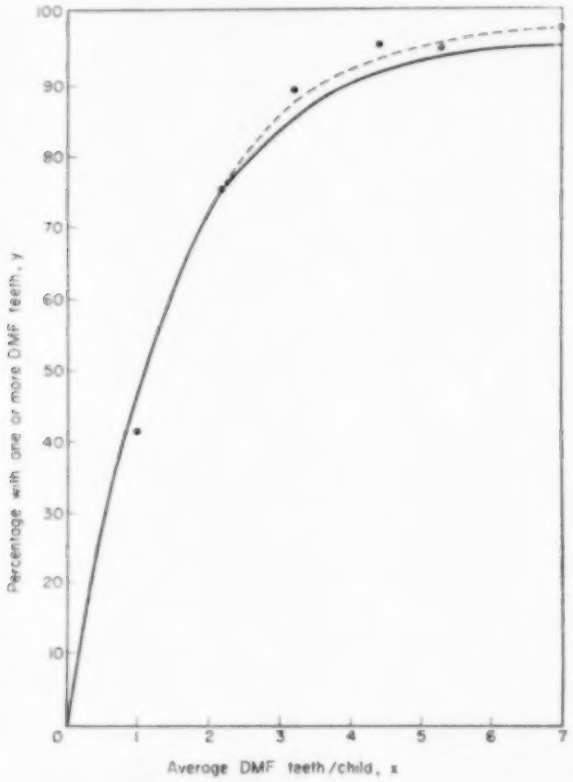

FIG. 1. Australian data; DMF teeth per child and percentage with one or more carious teeth. ——— Curve based on Knutson's equation $97-y=97\ (0{\cdot}524)^x$. - - - - - - - - Curve based on equation $100-y=100\ (0{\cdot}521)^x$. ● Australian data (BARNARD, 1956).

the group with dental caries reached 90 per cent at 8 years in Australia in contrast to 12–13 years in the Hagerstown figures from the U.S.A. The steep increase in the proportion of Australian children with one or more carious teeth makes it difficult to obtain an accurate estimate of the average DMF for children much beyond 8 years of age from the conversion table based on $97-y=97\ (0{\cdot}524)^x$.

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If different values for K are chosen instead of 97, and the $K-y$ values so obtained are plotted on semi-log paper against x (i.e. the observed average DMF teeth per child) the line which best fits the Australian data is that obtained with $K=100$ (Fig. 2).

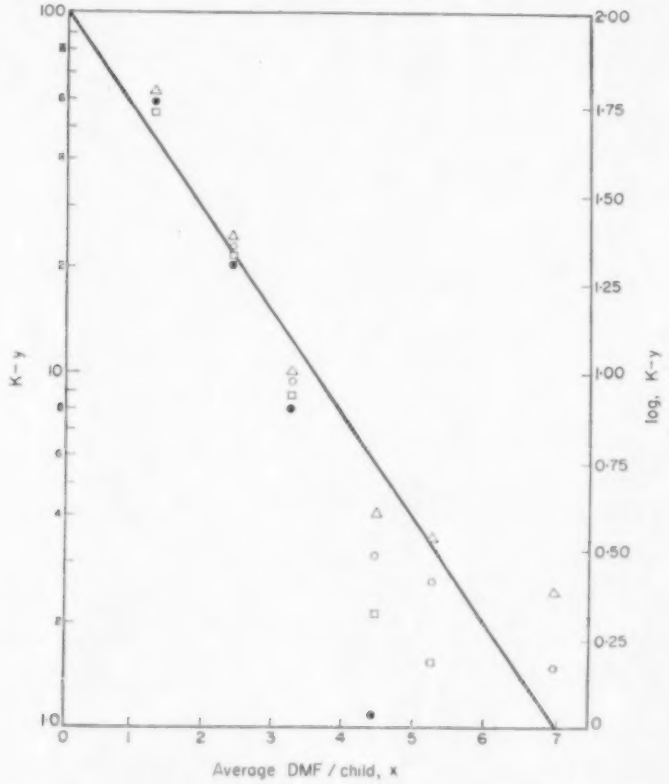

FIG. 2. Australian data; relation between averaged DMF per child and log ($K-y$).
△, $K=100$; ○, $K=99$; □, $K=98$; ●, $K=97$.

Reading from the graph for $K=100$, when $K-y=2$, it was found that $x=6$. Substituting these values in the equation $100-y=100\,B^x$ we have $2=100B^6$ and $B=0{\cdot}521$. This method of obtaining both K and B is the same as that used by KNUTSON (1944). Hence the Australian data are better described by the equation $100-y=100\,(0{\cdot}521)^x$ and a table to convert the percentages of children with one or more DMF teeth per child is included in Table 1.

Using this table to estimate the average DMF teeth per child for 6, 7, 8 and 9 year-olds from the percentage with one or more DMF teeth, the values so obtained, when plotted against age, fall on a straight line which on extrapolation gives a good approximation to the observed average number of DMF teeth per child for higher ages (Fig. 3).

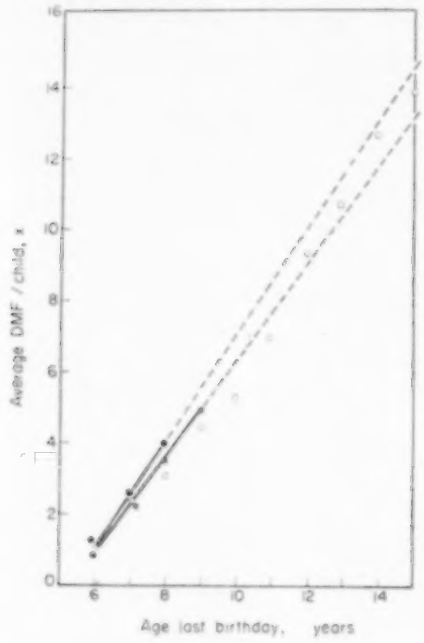

FIG. 3. Australian data; average DMF teeth per child by age. ○, Observed values (BARNARD, 1956); ⊙, Values estimated using equation $100-y=100\ (0{\cdot}521)^x$; ●, Values estimated using equation $97-y=97\ (0{\cdot}524)^x$; -------- Extrapolation from estimated values at 6, 7, 8 and 9 years.

TABLE 3. OBSERVED AND ESTIMATED DMF PER CHILD FOR AUSTRALIAN CHILDREN

	Age last birthday (years)									
	6	7	8	9	10	11	12	13	14	15
Percentage with one or more DMF teeth	41·3	76·5	89·5	95·9	93·4	97·5	99·0	99·6	99·4	100
Average DMF per child:										
1. Observed	0·99	2·31	3·22	4·44	5·28	6·98	9·32	10·70	12·78	13·91
2. Estimated by Knutson's equation and by extrapolation	0·86	2·41	3·97	5·60	7·20	8·75	10·30	11·85	13·30	14·80
Deviation from observed values	−0·13	+0·10	+0·75	+1·16	+1·92	+1·77	+0·98	+1·15	+0·52	+0·89
3. Estimated by equation $100-y=100\ (0{\cdot}521)^x$ and by extrapolation	0·80	2·20	3·40	4·85	6·30	7·75	9·20	10·60	11·95	13·35
Deviation from observed lines	−0·19	−0·11	+0·18	+0·41	+1·02	+0·77	−0·12	−0·10	−0·80	−0·56

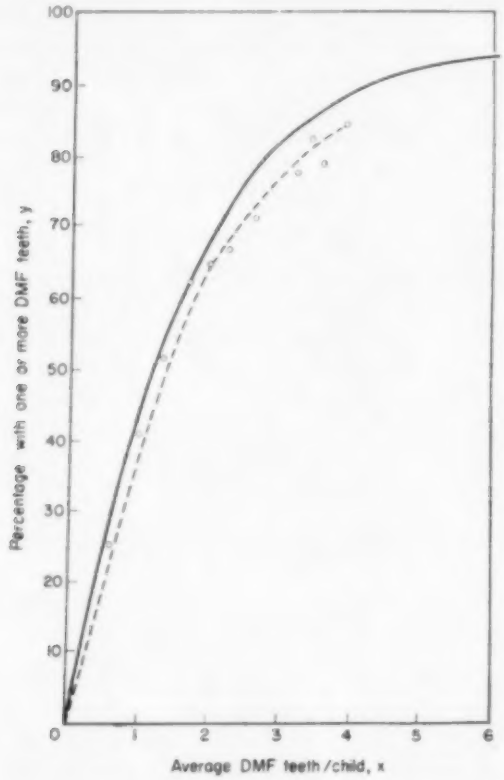

FIG. 4. Indonesian data; DMF teeth per child and percentage with one or more carious teeth. ——— Curve $97-y=97\ (0{\cdot}524)^x$; -------- Curve $93-y=93\ (0{\cdot}54)^x$; ○, Indonesian data (SOERIA SOEMANTRI, 1959).

TABLE 4. OBSERVED AND ESTIMATED AVERAGE DMF PER CHILD FOR INDONESIAN CHILDREN

	Age last birthday (years)										
	6	7	8	9	10	11	12	13	14	15	16
Percentage with one or more DMF teeth	25·8	41·3	51·9	62·3	65·1	67·4	71·4	78·4	79·9	82·8	85·2
Average DMF per child:											
1. Observed	0·54	0·96	1·27	1·64	1·91	2·21	2·58	3·14	3·51	3·35	3·80
2. Estimated from Knutson's equation	0·48	0·86	1·19	1·59	1·72	1·84	2·07	2·56	2·69	2·97	3·26
3. Estimated from equation $93-y=93\ (054)^y$	0·65	1·05	1·45	1·84	2·0	2·13	2·40	3·00	3·20	3·55	3·80

The smaller and random deviations from Barnard's observed values, in contrast to the larger and consistently overstated values derived from Knutson's table, indicate that this equation gives a better estimate for Australian children than does Knutson's equation (Table 3).

The theoretical curve $100-y=100\ (0{\cdot}521)^x$ is drawn in Fig. 1 and shows the adequacy of fit to Barnard's observations.

Indonesian children

The use of Knutson's equation, with $K=97$, gives reasonably good conversion data up to 10 years of age, when 65 per cent of the group have one or more DMF teeth. Above this level the differences between the observed values for DMF per child and those obtained from the conversion table exceed 10 per cent of the observed values. In addition the estimated DMF values obtained from the conversion table are consistently less than the observed values for each age group to 16 years of age. These irregularities are shown in Table 4 and Fig. 4.

Other values of K were examined and the equation with $K=93$ was found to be the one which most satisfactorily described the data. The constant B was calculated to be 0·54.

The adequacy of fit of the equation $93-y=93\ (0{\cdot}54)^x$ is shown in Fig. 4. Both Knutson's and this equation fit the data quite closely up to 10 years of age, but at higher ages the latter gives superior results.

Formosan children

The data for all Formosan children examined are shown in Table 5. In Table 6 the children drinking low fluoride-containing water are grouped together and, because the proportion of boys and girls in the groups were unequal, the average DMF per child values have been corrected to give mean DMF values for equal numbers of boys and girls at each age. A comparison of the observed corrected values for DMF per child with those derived from equations with different values of K showed that the closest estimates were obtained by using $K=100$.

TABLE 5. OBSERVED AND ESTIMATED AVERAGE DMF PER CHILD FOR FORMOSAN CHILDREN

	Age last birthday (years)		
	8	10	11
Percentage with one or more DMF teeth	67	54·4	55·4
Average DMF teeth per child:			
1. Observed	1·38	1·22	1·44
2. Estimated from Knutson's equation	1·82	1·28	1·31
3. Estimated from equation $100-y=100\ (0{\cdot}521)^x$	1·72	1·23	1·26

Data include all children examined in these age groups in Formosa and the Pescadores. Water supplies contained fluoride ion up to 1·6 p.p.m.

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TABLE 6. OBSERVED AND ESTIMATED AVERAGE DMF PER CHILD FOR FORMOSAN CHILDREN USING LOW FLUORIDE-WATER (LESS THAN 0·4 p.p.m. FLUORIDE)

	Age last birthday (years)		
	8	10	11
Number of children	211	138	185
Percentage with one or more DMF teeth	72	67	83
Average DMF per child:			
1. Observed (adjusted for unequal numbers of boys and girls)	1·62	1·67	2·75
2. Estimated from Knutson's equation	2·10	1·82	3·00
3. Estimated from equation $100-y=100\,(0{\cdot}521)$	1·95	1·70	2·77

	Age last birthday (years)					
	8		10		11	
	F	M	F	M	F	M
Number in group	76	135	57	87	83	102
DMF teeth	144	180	87	142	253	249
DMF per child	1·90	1·34	1·70	1·63	3·05	2·44
Mean by age	1·62		1·67		2·75	

However, conversion data for all the Formosan children aged 8, 10 and 11 years (Table 5) are as satisfactory for $K=100$ as for $K=97$. (Differences in DMF values for boys and girls and unequal numbers of each in these groups were insignificant and have been omitted.)

DISCUSSION

Barnard's data for the prevalence of dental caries in Australian children are better expressed by an equation which recognizes that up to 100 per cent may be found to have evidence of dental caries ($K=100$) than by Knutson's equation which takes 97 per cent ($K=97$) as the upper limit. However, these data fall within the range of variation found in the U.S.A. (KNUTSON, 1958) and appear to lie between those of Grand Rapids, Michigan before fluoridation (1944–45) and Woonsocket, Rhode Island (1945), two cities with high caries prevalence tending to affect 100 per cent of the children by age 16 years.

There are indications that the caries prevalence in Formosa may, similarly, be better expressed by an equation using $K=100$. It is worthy of note, however, that

Knutson's equation was equally satisfactory when applied to the data for all the children. However, the number of age groups is too small to enable more than this general observation to be made.

In contrast, the Indonesian data required a lower value, 93 per cent (K=93), as the upper limit of the percentage of children ultimately to be affected by dental caries, although Knutson's equation provided equally good estimates of the mean DMF up to the age when about 65 per cent of children have one or more DMF teeth.

The estimated DMF values obtained by using Knutson's equation are, as often as not, within 10 per cent of the observed values obtained from full examination, charting, and statistical tabulation, an amount of variation which exists in the reported findings from examinations in different localities within the United States of America (KNUTSON, 1958).

The data presented here relate to three different countries, with distinct genetic groups, and with high and low levels of caries prevalence, and is striking confirmation of the validity of the general relationship described by Knutson between the percentage of children (by age) with one or more DMF teeth and the average number of DMF teeth per child. They also confirm the usefulness, for public health purposes, of the conversion table derived from his equation.

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SHORT COMMUNICATION

AN X-RAY DIFFRACTION PATTERN FROM HUMAN ENAMEL MATRIX

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WHILE THE ENAMEL MATRIX has been defined as a eukeratin (BLOCK, 1937) on the basis of the arginine:lysine:histidine ratios, the presence of about 5 per cent hydroxyproline in the analyses reported by STACK (1954), HESS and LEE (1954) and BATTISTONE and BURNETT (1956) led GELLER (1958), among others, to suggest that the enamel protein might be a collagen rather than a keratin. This possibility is emphasized by investigations into invertebrate collagens (GROSS, SOKOL and ROUGVIE, 1956; PIEZ and GROSS, 1959), where cystine is present and the proportion of hydroxyproline is less than in mammalian collagens. Recently, however, amino-acid analyses of enamel proteins have been reported that differ from those previously published. EASTOE (1960) found no hydroxyproline in dissected and decalcified human foetal teeth and concluded that the organic matrix could not be compared with any known protein. PIEZ (1960) also reported no hydroxyproline in protein isolated from unerupted human third molar teeth and inferred that the enamel matrix was not a collagen.

Crystallographic identification of the enamel matrix has proved difficult because of the sparseness of the protein and the disordered state of the organic framework once the inorganic salts have been removed. However, as a result of the surgical removal of unerupted deciduous teeth from an infant with a congenital pigmented epulis (MITCHELL and TALMAGE READ, 1960), specimens of decalcified enamel of sufficient size to permit the examination of oriented samples by X-ray diffraction have become available.

The unerupted maxillary molar tooth, removed from a 3-months-old infant, was fixed in formol–saline, decalcified in ethylenediamine tetra-acetic acid at pH 7, dehydrated in alcohol, and embedded in paraffin wax at 60°C. Microtome sections of the embedded tooth (Fig. 1) showed areas in which the enamel matrix remained intact.

For examination by X-ray diffraction, sections from 25–75 μ thick were cut from the whole embedded tooth and dewaxed by repeated washing with xylene. The sections were dried on a glass block and specimens of the dentine and enamel matrices were removed with a razor blade. Each specimen was oriented under the microscope on a thin collodion film supported across a slot in a brass holder. In some cases two or three fragments of the enamel matrix were mounted one on top of the other

with the prism axes parallel. Diffraction photographs were taken at 4 cm specimen-to-film distance in a flat-film camera using nickel-filtered CuKα radiation collimated by a glass capillary.

Specimens of dentine removed from the area marked D in Fig. 1 and mounted with the axis of the tubules mounted vertical gave an oriented diffraction pattern suggesting collagen. The characteristic wide-angle reflexion at 2·8 Å and the prominent side-chain reflexion at about 11 Å could be seen after exposure times of only 6 hr (Fig. 2). On the other hand, even after prolonged exposures up to 80 hr, no reflexions suggesting collagen could be found in the photographs of the enamel specimen taken from the area marked E in Fig. 1 and mounted with the prism axis vertical. Instead, a sharp reflexion at 4·65 Å, preferentially oriented on the meridian, appears in the diffraction diagram, together with a fainter meridional arc at 4·1 Å and a diffuse halo at about 10 Å (Fig. 3).

The absence of a wide-angle diffraction pattern indicative of collagen in the enamel matrix after exposure times greatly in excess of those required to produce a clear collagen pattern from the dentine matrix shows that little or no collagen is present in enamel. It could be argued that any collagen in the enamel might be in too fine a state of division, or too disordered, to form an adequate diffracting material, but this seems unlikely in view of the easily recognized collagen pattern obtained from the dentine matrix and the clarity of the diffraction pattern that is actually obtained from the enamel matrix.

The diffraction pattern that has been obtained from this one specimen of enamel matrix suggests protein in the supercontracted state. The sharp meridional reflexion at 4·65 Å is characteristic of the cross-β configuration (RUDALL, 1946) found in proteins after thermal or acid treatment (ASTBURY, BEIGHTON and PARKER, 1959), and the enamel protein might therefore be classified as a member of the k-m-e-f group. However with a decalcified tooth that has been heated to 60°C during embedding, it is not possible to decide whether the enamel protein occurs naturally in the supercontracted state, as is the case, for example, with the egg-stalk of the lace-wing fly *Chrysopa* (PARKER and RUDALL, 1957); or whether the protein has been supercontracted by the heat treatment. The faint reflexion at 4·1 Å on the meridian suggests fat or lipid, and this may be associated with the enamel protein or may have been introduced during the embedding process. There are some resemblances between the diffraction pattern of the enamel protein and that of trout-sperm flagella (PAUTARD, 1960).

Although this specimen of insoluble enamel matrix might be classified tentatively as a member of the k-m-e-f group of fibrous proteins, there is no evidence as yet for the configuration of the protein (or proteins) in the native state.

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PLATE I OVERLEAF

FIG. 1. Longitudinal section of the decalcified unerupted molar. Stained with haematoxylin and eosin. ×12.

FIG. 2. X-ray diffraction pattern from the area of dentine matrix marked D in Fig. 1. Axis of tubules vertical. 6 hr exposure.

FIG. 3. X-ray diffraction pattern from the area of enamel matrix marked E in Fig. 1. Prism axis vertical. 20 hr exposure.

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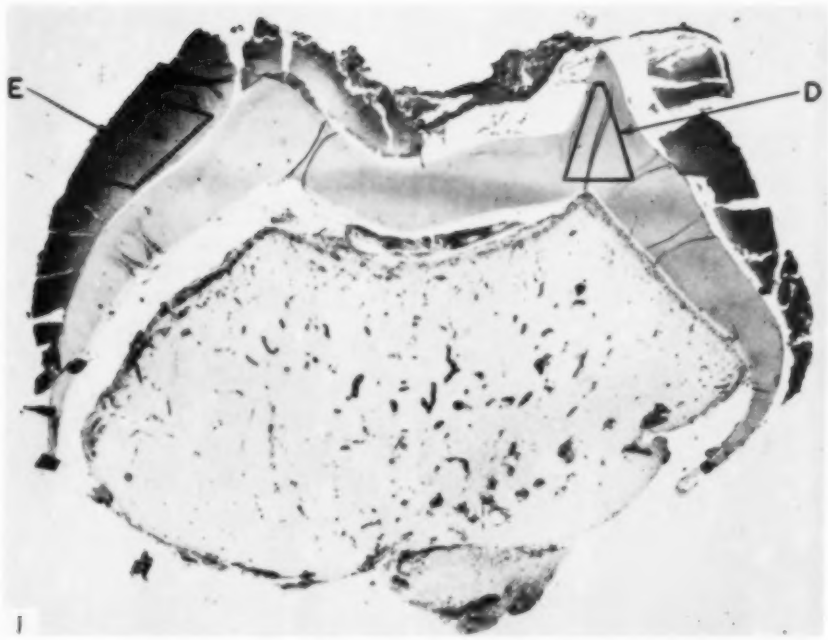

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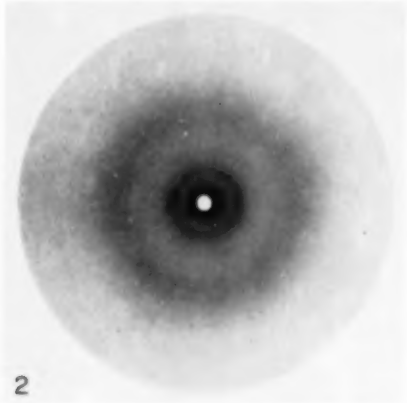

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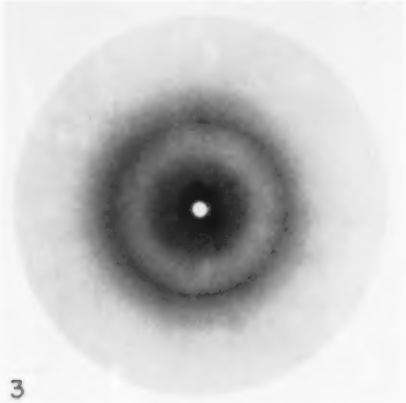

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PLATE 1

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TISSUE REACTIONS FOLLOWING INTRAMUSCULAR INJECTION OF PENICILLIN PREPARATIONS

AN EXPERIMENTAL STUDY

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Abstract—An investigation into the tissue reactions of white rats injected with two penicillin preparations is described. 0·3 ml of one of the solutions under test was injected into the gluteus muscle on one side and 0·3 ml of physiological saline solution was injected as control on the opposite side.

Histologic examination showed that both preparations damage the fat, the muscle and, to some extent, the vessels. After approximately 30 min inflammatory reactions were observed in the fat and later in the muscle, where the muscle fibres degenerated and became separated from the surrounding healthy tissue by inflammatory cells.

At the site of the control injections no reactions could be seen excepting a slight increase of the inflammatory cells during the first few hours following injection.

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INTRODUCTION

PENICILLIN is still the most frequently used antibiotic, partly because side reactions after administration of even large doses are frequently very slight. The most common side effects are exanthemata of urticarial type and a steadily increasing resistance of bacteria. In connexion with the introduction of prolonged action penicillin, local reactions such as pain and tenderness at the point of injection have been common. How the living tissue reacts after the injection of a penicillin preparation has been the subject of some shorter investigations, and these seem primarily to have been concerned with preparations of the more prolonged action types (GALLEGO, 1955–56; SEIFTER *et al.*, 1951; DICKISON, HOEKSTRA and TISCH, 1953–54; KRUGMAN and POWELL, 1956; and others).

In an experimental study BJÖRLIN and GUSTAFSON (1958) showed that an intramuscular injection of dibenzylethylenediamine penicillin (DBED pencillin) had a deleterious effect on living tissue in white rats. The results of this investigation suggested an investigation of other penicillin preparations.

The aim of this study was to test, in white rats, the effect on the tissues of two penicillin preparations used at the Department of Oral Surgery at the Royal Dental School, Malmö.

METHOD

The experiments have been carried out on white rats weighing 150–200 g. The method was the same as that used by BJÖRLIN and GUSTAFSON (1958). A short recapitulation of this will be given here.

0·3 ml of the penicillin solution under test was injected in the gluteus muscle on one side. In the corresponding muscle on the other side 0·3 ml of physiological saline solution was injected as control. The animals were divided into two groups. The first group was tested with a proprietary preparation called Streptopenin and having the following composition:

Procaine benzyl penicillin	0·15 g
Dihydrostreptomycin sulphate equivalent to dihydrostreptomycin base	0·25 g
Procaine hydrochloride	0·02 g
Sodium citrate (tribasic)	0·015 g
Phenylcarbinol, polysorbate 80 U.S.P. XIV, lecithin, sodium hydroxide to pH about 7·3	q.s.
Sterile water	ad 1 ml

The second group received Suspenin:

Procaine benzyl penicillin	0·3 g
Procaine hydrochloride	0·02 g
Sodium carboxymethylcellulose, tribasic sodium citrate, sodium sulphite, methyl parahydroxybenzoate, polysorbate 80 U.S.P. XIV	q.s.
Sterile water	ad 1 ml

The animals were killed after varying intervals: 30 min, 1 hr, 2 hr, 4 hr and so on up to 30 days. The muscle containing the depot was dissected out, fixed in formalin, embedded, sectioned and stained with haematoxylin and eosin.

RESULTS

The microscopical examination showed more or less pronounced tissue changes after injection of both Streptopenin and Suspenin. The changes were of the same type and therefore photomicrographs are shown only from changes after Streptopenin injection.

Vascular changes

Streptopenin. Ruptures of the walls of vessels were observed. In tissues removed 2 hr after injection a large haemorrhage could be seen (Fig. 1). In tissues removed 3 days after injection necrosis and thrombosis in the smaller vessels could be seen in some cases.

Suspenin. Changes in the vessels after the injection of Suspenin were less severe than after injection of Streptopenin. In some cases thrombosis was found only in the smaller capillaries.

Cellular changes

Streptopenin. 2 hr after injection there was an increase of inflammatory cells. After 1 day there was a large number of polymorphonuclear leukocytes, lymphocytes and plasma cells. In specimens removed 2 days after injection, granulation tissue had begun to form and after 8 days granulation tissue covered the injured muscle.

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Suspenin. As in tissues from experiments with Streptopenin, there was an increase of inflammatory cells a short time (1 hr) after injection of Suspenin. The number of inflammatory cells increased rapidly, first in the fat and later in the muscle. After 1 day there was a large number of polymorphonuclear leukocytes, lymphocytes and plasma cells. 3 days after injection the first fibroblasts appeared and after 6 days a well-developed granulation tissue could be seen.

Changes in the fat

Streptopenin. Fat cells degenerated at an early stage. After 1 hr inflammatory cells had invaded the degenerating tissue and after 1 day there was a distinct limit between normal and degenerated fat. After 6 days granulation tissue appeared but there was no sign of encapsulation of the depot, as was seen 7 days after injection of DBED-penicillin (BJÖRLIN and GUSTAFSON, 1958).

Suspenin. In tissue where Suspenin was injected there was a similar reaction: inflammatory cell infiltration 1 hr after injection of the solution. The quantity of these cells increased but the tissue reaction seemed not to be so severe as after injection of Streptopenin.

Changes in the muscle

Streptopenin. Changes in the muscle were seen as early as 1 hr after injection. At this stage there were only occasional inflammatory cells but the muscle fibres showed degenerative changes with altered staining characteristics (Fig. 2). These changes were seen only in the neighbourhood of the depot. 1 day after injection there was a massive infiltration of lymphocytes, polymorphonuclear leukocytes and plasma cells (Fig. 3), which had penetrated the separated and degenerated muscle fibres (Fig. 4). In tissue removed 6 days after injection of Streptopenin only the pale contours of the muscle fibres could be seen (Fig. 5). 8 days after the injection only small remnants of the muscle fibres remained in the granulation tissue (Fig. 6).

Suspenin. 8 hr after the injection there was an infiltration of inflammatory cells in the muscle. These cells were packed in between the muscle fibres and in the fibres there were signs of degeneration. After 2 days there was a mass of inflammatory cells of the same type as described earlier, and the muscle fibres were separated from their connexion with the remaining muscle tissue. These fibres, which showed changed staining characteristics and loss of the nuclei, were gradually destroyed by the inflammatory cells. They were separated and, at least in one place, only the contours of the fibres could be seen.

Controls

Upon injections of physiological saline solution a slight increase of the inflammatory cells was observed only during the first few hours, probably due to the injection trauma.

DISCUSSION

The results show that the penicillin preparations used have a deleterious effect on living tissue of white rats. Of the two preparations, Streptopenin seemed to damage the tissue to a greater extent than Suspenin. The damage was not as serious,

however, as that obtained with an earlier investigation (BJÖRLIN and GUSTAFSON, 1958) on DBED-penicillin.

The penicillin preparations used here contained procaine penicillin. After 10 days the depot was resorbed, and it was macroscopically impossible to find where it had been situated. In spite of the relatively large quantity injected, no obvious general reactions were observed.

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FIG. 1. *Streptopenin.* Specimen 2 hr after injection of 0·3 ml. To the left is a large haemorrhage. Haematoxylin and eosin. ×36.

FIG. 2. *Streptopenin.* Degenerative changes in the muscle fibres with altered staining characteristics. Specimen 2 hr after injection. Haematoxylin and eosin. ×96.

FIG. 3. *Streptopenin.* Specimen 1 day after injection. Inflammatory cells have invaded the degenerated muscle fibres. Haematoxylin and eosin. ×96.

FIG. 4. *Streptopenin.* Specimen 1 day after injection. A partly destroyed muscle fibre invaded by inflammatory cells. Haematoxylin and eosin. ×360.

FIG. 5. *Streptopenin.* Specimen 6 days after injection. Only pale contours of the muscle fibres can be seen. Haematoxylin and eosin. ×360.

FIG. 6. *Streptopenin.* Specimen 8 days after injection. In the granulation tissue there are numerous rests of more or less degenerated muscle fibres. Haematoxylin and eosin. ×360.

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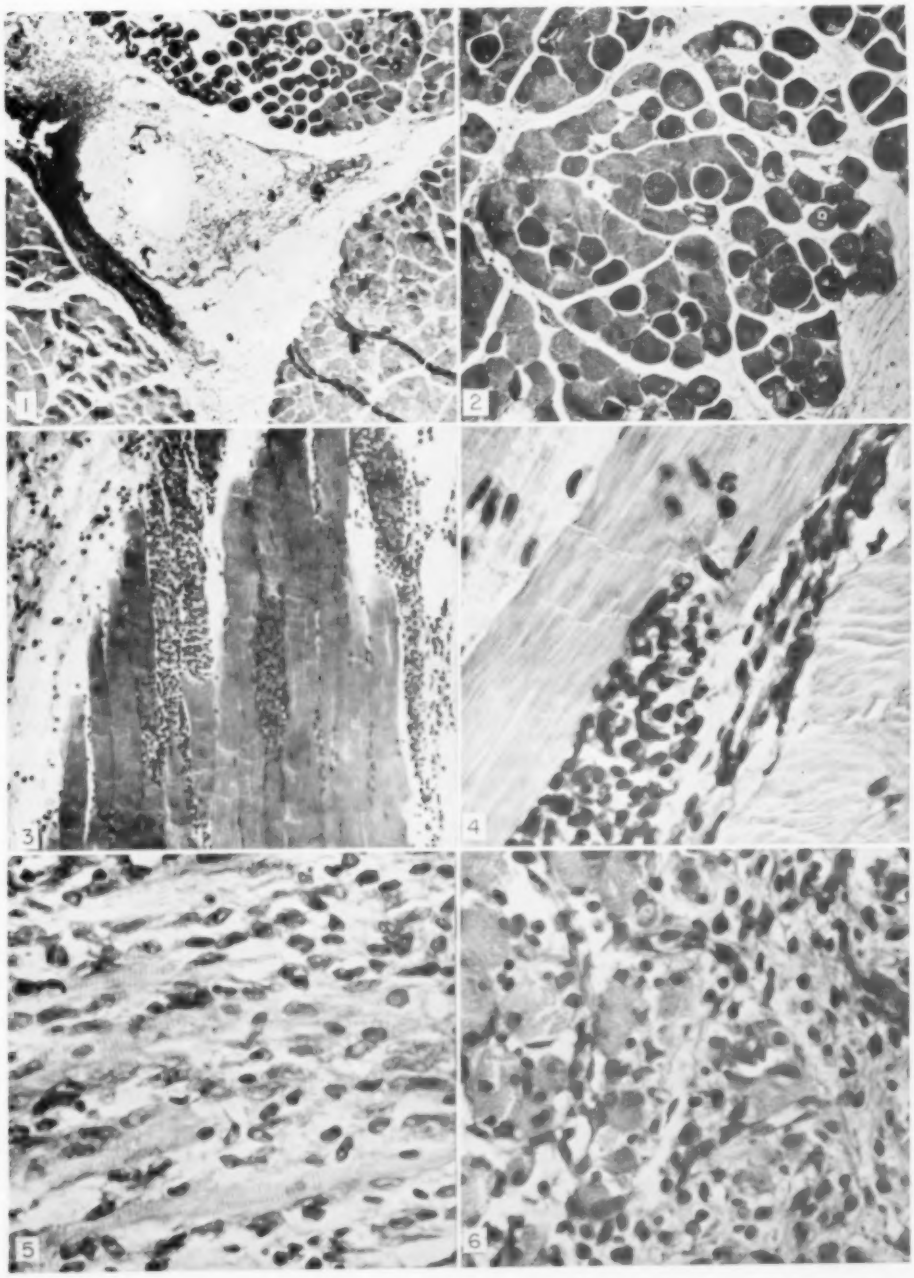

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THE RELATION OF RADIATION DOSE TO RADIATION DAMAGE IN THE MANDIBLE OF WEANLING RABBITS

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Abstract—Weanling rabbits injected with a single dose of ^{90}Sr Cl in amounts from 100 to 1000 μc/kg were killed at intervals up to 180 days. Autoradiographic and dosimetric studies were performed on the mandibles of one side and histological studies on the other. The pattern of ^{90}Sr retention in the teeth differed in rabbits given 600 μc/kg or more from those given 100 μc/kg or less owing to retardation of tooth eruption and growth in the former group. Dose-rates were measured along two lines in the mid-vertical plane of the mandible and variations in dose-rate with time after injection for rabbits of higher and lower injected doses were recorded. Variation of accumulated dose with time after injection was also calculated. Severe and early changes in tooth growth occurred after the higher injected doses, teeth becoming narrower, retarded or arrested as to growth in length, and solid. Alveolar bone ceased to grow, but 2–3 months after injection pleomorphic osteoblastic proliferations arose on the surface of sockets and by 6 months resorption of tooth and bone accompanied growths regarded as osteosarcoma. No tumours arose from dental tissues. The relationship between dosage and histological changes is discussed.

INTRODUCTION

IT IS NOW well recognized that wherever calcium is present in the body strontium will also be found. No natural source of calcium is free from strontium and the chemical properties of the two elements are so similar that the body treats them very similarly. Thus, when radioactive isotopes of strontium are administered, they will be found in all calcified tissues unless these are so remote from circulating blood that the radioactive strontium has no access. Once deposited, it is inevitable that the beta-particle emitting isotopes, ^{89}Sr and ^{90}Sr, will cause local irradiation of the tissues which contain them and also of small volumes of tissue immediately adjacent, the size of the latter depending on the range of the particular beta-particles involved. In irradiated tissues cells may be killed and cell division may be interfered with so that growth and organization may be disturbed. Tumours may also result. The degree of effect depends, amongst other things, on the concentration of isotope in the tissues and how long it is retained there. In the jaws of an animal like the rabbit, bearing teeth which are continuously calcifying and erupting, there are possibilities of quite complex disturbances of what is normally a highly integrated succession of tissue changes.

A preliminary account has already been given of the general radiological changes in the jaws of both young and old rabbits following the administration of large

amounts of ^{90}Sr, together with an autoradiographic study showing the pattern of ^{90}Sr distribution (HOLGATE, 1959). This pattern of distribution is greatly affected by the amount of ^{90}Sr administered since large amounts cause considerable damage and prevent the normal eruption of the teeth while smaller amounts may give rise to lesser degrees of damage from which there is recovery. It is also known that one of the most frequent sites for the development of osteosarcoma following ^{90}Sr adminstration is the jaws (OWEN, SISSONS and VAUGHAN, 1957; OWEN and VAUGHAN, 1959a).

The present investigation was planned to study the effect of giving a single intravenous injection of ^{90}Sr to weanling rabbits at a level known to be highly carcinogenic to the jaws and at other somewhat lower levels. In particular an attempt was made to relate the radiation damage seen on histological examination to absorbed radiation dose in rads. In the course of the investigation it became evident that certain of the changes noted in teeth, particularly following the smaller injected doses of ^{90}Sr, were similar in character to those seen in old control rabbits which were being studied in relation to an analysis of the relation between radiation dose and radiation damage in the jaws of old animals. The full results of this latter study will be reported in detail later but discussion of the changes seen in the control old rabbits has been necessary here since it is possible that some of the effects of ^{90}Sr in the weanling might be interpreted as signs of "ageing"—an effect often attributed to radiation.

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EXPERIMENTAL METHODS

(i) *Rabbits*

Dutch rabbits aged 5–8 weeks were injected with ^{90}Sr Cl in amounts varying from 100 to 1000 μc/kg as previously described (OWEN *et al.*, 1957). Some were allowed to survive and others were sacrificed for special studies at different time intervals. The jaws after dissection were radiographed. In the case of the series of rabbits used for special dosimetric studies, which were given 100 or 600 μc/kg of ^{90}Sr, one mandible was fixed in alcohol and used for the preparation of thick autoradiographs while the other was fixed in formol saline for histological studies. Additional histological material and sections for the preparation of thin autoradiographs were obtained from rabbits in which half of the jaw was used to estimate ^{90}Sr retention (HOLGATE and MOLE, in preparation) and from rabbits in which other studies of the effect of ^{90}Sr on the skeleton were being made (OWEN *et al.*, 1957; OWEN and VAUGHAN, 1959a).

(ii) *Measurements of radiation dose*

The method of determining the dose-rate from autoradiographs of "thick sections" of tissue containing radioactive material, has already been described in detail (SINCLAIR *et al.*, 1956; OWEN and VAUGHAN, 1959b). Essentially the same method has been used in the present case.

The right mandible was cut in two pieces at the junction of the incisor tooth and the first premolar. The portion containing the cheek teeth was embedded in methyl methacrylate monomer and cut on a milling machine through the middle of

the teeth and mandible in a plane through the vertical axes of the teeth. In this case the complete half of the bone and teeth constituted the "thick section" from which autoradiographs were obtained. The surfaces of the two halves were exposed in contact with an Ilford ordinary fine grain photographic plate. The blackening of the autoradiograph gives a picture of the distribution of radiation dose-rate in the plane of the cut surfaces, i.e. in the plane through the middle of the teeth and mandible. The photographic density was measured over an area 50 μ square using a microdensitometer. The radiation dose-rate was calculated from a calibration curve of dose against density previously obtained for these plates, using a ^{90}Sr standard whose dose-rate was known. The total dose-rate was obtained by adding together the contributions from both halves.

Measurements were made on two series of rabbits, one given 100 and the other 600 μc of ^{90}Sr/kg body weight. The rabbits were killed at 8 and 24 hr, 9, 16, 30, 60, 90 and 180 days after injection. Three animals were measured at each time interval.

(iii) *Histological methods*

Histological examinations were carried out generally on the left mandibles, both in the region of the premolars, molars, and of the incisors. Maxillary teeth were also examined in some cases.

Specimens were demineralized in EDTA after formalin fixation, and embedded in celloidin or paraffin. They were stained with haematoxylin and eosin and van Gieson's stain.

The material examined histologically consisted of fifteen controls from weanling to 4½ years of age; twenty-one rabbits which had 100 μc/kg at 5–8 weeks; three which had 200 μc/kg; thirty-four which had 600 μc/kg; and three which had 1000 μc/kg.

RESULTS

(i) *Dosimetric measurements*

The pattern of ^{90}Sr retention in the teeth and jaws of young rabbits has already been described (HOLGATE, 1959). Immediately after injection the ^{90}Sr is taken up in the calcifying regions of bone, in the calcifying dentine which surrounds the tooth pulp chamber, in recently formed cementum and in recently formed enamel at the base of the teeth. With time after injection there is some resorption of the ^{90}Sr from the bone, and the dentine containing the ^{90}Sr moves towards the occlusal surfaces where it is eventually worn away by attrition as the normal development of the tooth takes place. It should be mentioned at this point that the pattern of ^{90}Sr retention in the teeth of rabbits given 600 μc/kg or more is different from that in rabbits given 100 μc/kg or less. At the higher injected dose level there is considerable radiation damage, the normal eruption of the teeth is inhibited, and the movement of dental tissues containing ^{90}Sr towards the occlusal surfaces and its removal there are very much slowed down.

The above picture of the uptake and retention of ^{90}Sr has been confirmed by the autoradiographs of the thick sections (or two halves) of the teeth and mandible obtained for dosimetry measurements and is illustrated in Figs. 1 and 2. Fig. 1 is

an autoradiograph of half of the lower mandible of a rabbit given 100 μc/kg at the age of 5–8 weeks and killed 24 hr later. The resolution of the autoradiograph of a section of this thickness (about 2 mm), although not good, is sufficient to show uptake of ^{90}Sr in the regions of bone and dentine described above. Fig. 2 shows a similar autoradiograph from a rabbit killed 30 days after injection. The movement of the dentine containing the ^{90}Sr towards the occlusal surfaces of the teeth is clearly illustrated. It will also be appreciated from Figs. 1 and 2 that the ^{90}Sr deposits are numerously distributed throughout the bone and teeth. This, together with the long range of the beta particle from ^{90}Sr + ^{90}Y results in widespread irradiation of all the tissues of the bone and teeth and, as will be seen from the results, the variation in dose-rate throughout the tissue is not so great as might have been expected at first sight.

The variation in dose-rate was measured along two lines, shown in Figs. 1 and 2, in the mid-vertical plane of the jaw. One line was drawn horizontally through the centre of the teeth and the other vertically through the bone and soft tissues between the first and second molar teeth. The highest dose-rates were found in the middle of the deposit in the alveolar bone and in the middle of the deposit in the dentine where they were of approximately the same value. The minimum dose-rates in the region of bone and soft tissues, were received in the middle of the marrow spaces between the bases of the teeth and it is these values which are shown in Fig. 3.

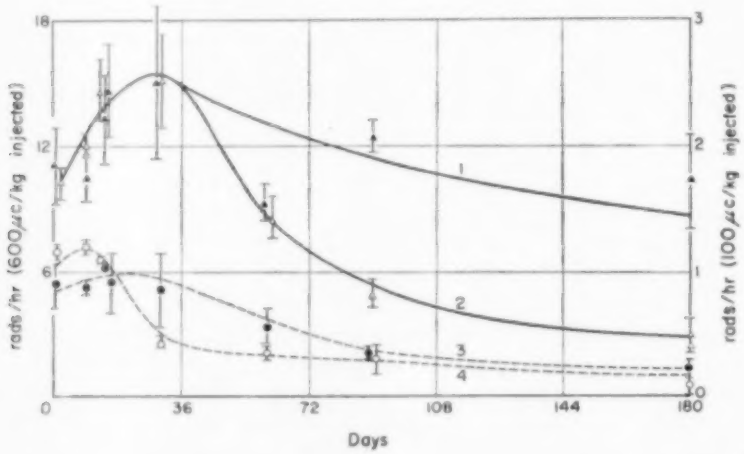

FIG. 3. Variation in dose-rate with time after injection. Curves (1), ▲, and (3), ●, are the maximum and minimum dose-rates for rabbits injected with 600 μc of ^{90}Sr/kg, scale on left. Curves (2), △, and (4), ○, are the maximum and minimum dose-rates for rabbits injected with 100 μc of ^{90}Sr/kg, scale on right.

Immediately after injection the lowest dose-rates were received at the occlusal ends of the teeth. The variation in the maximum and minimum dose-rates in the teeth and mandible during the period up to 6 months after injection is shown in Fig. 3.

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Each point is the average of measurements made on three rabbits. The errors shown are the standard deviations.

There are several interesting features about the results shown in Fig. 3. The maximum dose-rate increases to its highest value at about 30 days, and then falls off. This is in agreement with previous results (HOLGATE, MOLE and VAUGHAN, 1959) which reported an increase in the retained ^{90}Sr per gram of bone up to a maximum at 30 days. During the first 30 days after injection the maximum dose-rate for the rabbits given 600 μc/kg is about six times that in the rabbits given 100 μc/kg. After 30 days the dose-rates in the rabbits given the higher injected dose are relatively greater, due to the slowing down of eruption and lack of attrition of the teeth as a result of radiation damage which delays the loss of tissue containing the ^{90}Sr. The variation in dose-rate, or the ratio maximum dose-rate/minimum dose-rate throughout all the bone and dental tissues is not very great particularly up to 30 days when it is only a factor of about 2. Thereafter it increases to about 9 for the rabbits given 600 μc/kg and to about 5 for the rabbits given 100 μc/kg.

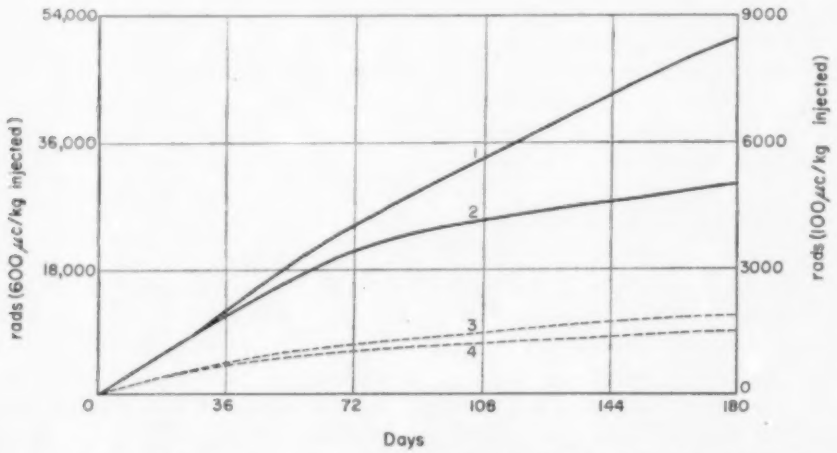

FIG. 4. Variation in accumulated dose with time after injection. Curves (1) and (3) are the maximum and minimum accumulated doses for rabbits injected with 600 μc of ^{90}Sr/kg, scale on left. Curves (2) and (4) are the maximum and minimum accumulated doses for rabbits injected with 100 μc of ^{90}Sr/kg, scale on right.

The accumulated doses at varying times after injection were calculated using the curves in Fig. 3 and these are shown in Fig. 4. The ratio maximum accumulated dose/minimum accumulated dose was not high. Its greatest value was at 6 months after injection where it was about 5 in the case of the rabbits given 600 μc/kg, and about 3·5 in the case of those given 100 μc/kg. Hence the variation in the accumulated dose throughout all tissues was not more than a factor of 5. The maximum accumulated dose was received in the middle of the deposit of ^{90}Sr in the dentine. The parts which consistently showed the most disturbed development, on the other hand, were

the developing bases of the teeth and the osteogenic connective tissue covering bone. These tissues received dose-rates and an accumulated dose only about 10 per cent higher than the minimum accumulated dose.

(ii) *Histology*

Injected dose of ^{90}Sr, 600–1000 μc/kg *at the age of* 5–8 *weeks.* Profound effects were produced on the development of dental tissues by administered doses of 600–1000 μc/kg ^{90}Sr. In the description of these changes the radiation doses quoted have only been calculated for rabbits receiving 600 μc/kg. A higher radiation dose approximately in proportion would be received following an initial administration of 1000 μc/kg.

Dental pulp and dentine. At the base of the premolar and molar teeth where the enamel organ is directed inwards towards the immature base of the pulp, it normally has adjacent to it a dense accumulation of small pulp cells which may by division and migration supply cells to the base of the pulp on the one hand and odontoblasts on the other. There is no interruption in the continuous sheet of cells in either direction (Figs. 5 and 7) and there is a gradation of cell forms between the densely crowded base of the pulp and spaced stellate cells of its more mature part.

At 24 hr and more clearly at 3 days after injection of ^{90}Sr there was evidence of damage to cells near to or continuous with the odontoblast row at a point where they had not yet become columnar (Fig. 9). Fragmented and pyknotic nuclei could be found and the cells had become separated from the internal enamel epithelium. When 600 μc/kg ^{90}Sr had been administered the accumulated dose in this area at 24 hr was about 160 rads and at 3 days 500 rads. At 9 days and more obviously at 14–16 days (2500 rads) there was a decreased density of cells at the same point and on the superficial side of it (Fig. 8). At 30 and 60 days there might be a wide gap next to the epithelium at this point with a deposit of eosinophil material which is not collagen (4400 and 7400 rads, Fig. 10). At 30 days there was also no longer a gradual change in character of pulp cells from the base towards the mouth but an abrupt change (Fig. 6). The immature pulp cells were separated from those more mature by a cell-poor zone and an arc-like arrangement of cells at the base of the older portion of the pulp. The beginning of this arrangement can already be seen 14 days after injection. At this level there is also an increased quantity of collagen fibres running in the long axis of the pulp. The pulp tissue superficial to this arc is chiefly that which was formed before or soon after injection, which has not moved at the normal rate towards the mouth, and which has not received substantial increments at its base. In this part the odontoblasts are fewer and shorter than in controls (Figs. 7 and 8).

The teeth do not altogether cease to grow and erupt for several weeks after injection, though they become greatly retarded. Mitoses continue near and among the recently differentiated odontoblasts and adjacent pulp cells and autoradiographs show that the ^{90}strontium-bearing zone of dentine moves somewhat towards the mouth. The bases of the teeth are also narrower than at the time of injection, as shown in radiographs, indicating that some new dentine has been added to the length of the tooth. While this addition gradually ceases, the dentine already formed

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at the time of injection continues to increase in thickness by apposition on its pulpal aspect. In the older part of the tooth this apposition is regular in arrangement, but in the more recently formed part it is often highly irregular (Figs. 6, 10). Some odontoblasts fail to retire as matrix is deposited and substantial deposits of matrix appear on the pulp side of many of them. Thus single or whole rows of odontoblasts may remain entrapped in the matrix, sometimes with capillaries also, and these cells subsequently die. Apposition of dentine continues until all the older part of the pulp has been replaced (Figs. 16, 17). This is not in itself abnormal: the abnormality here is its occurrence in a situation deep in the jaw owing to failure of continued eruption of the tooth. The immature part of the pulp remains as a nodule at the base of the tooth and does not afterwards proliferate. No neoplastic changes have been observed in it.

In the incisors the derangement is of the same character as in the cheek teeth with some differences. Early damage is seen among the pre-odontoblasts, with pyknosis and separation from the internal enamel epithelium at 24 hr and subsequent depletion of pulp cells in the area. The damage is more severe on the lingual or concave side. Disturbed dentine formation with inclusion of cells in the matrix may begin very early (before 9 days) on this side of the tooth, but the tooth does not cease growing in length for many months. There is in fact very active proliferation of pulp cells just anterior to the cell-poor area; and anterior to this, as some odontoblasts become embedded, others surviving or newly differentiated or the result of division clothe the pulp side of successive layers of matrix. The immature part of the pulp forms a spherical mass separated from the rest of the pulp by the cell-poor zone and displaced towards the lingual side (Fig. 11). Between the cell-poor zone on this side and the proliferating odontoblasts, rather dense bundles of collagen fibres, abnormal for the age, appear in the long axis of the tooth (Fig. 12). Rather similar fibres may be found in old control rabbits (e.g. 4 years). Although the incisor continues to grow and erupt, it becomes progressively narrower and this may result from lack of contribution of cells from the area first damaged or lack of migration of cells from the immature base of the pulp. Patches of collagenous matrix periodically appearing on the pulp side of the odontoblasts ultimately become incorporated in the dentine. If Hertwig's sheath becomes destroyed, the lingual dentine may become highly deranged with irregular outline and incorporation of cells, some of which may be cementoblasts from the periodontal membrane (Fig. 11).

An interesting feature is that the proliferation of pulp cells near and among the differentiated odontoblasts appears sufficient to promote considerable growth of the tooth in length (though not the maintenance of its normal width) without the normal participation of the most basal part of the pulp. In a specimen 4 months after injection, where the incisor has become extremely narrow, the sphere of immature pulp appears extruded beyond the base of the tooth, the diameter of which it exceeds, while growth of the tooth is apparently maintained by proliferation at the base of the calcified portion.

Cementum and periodontal membrane. Normally cementum does not form on the most basal parts of the cheek teeth, which are covered on their surface with dental

epithelium, but is deposited on these same parts after a time interval, during which they have moved towards the mouth. In the injected rabbits, movement of the teeth towards the mouth is greatly retarded, but the apposition of cementum is not prevented. Thus, comparison of the experimental tooth at 30 days after injection (4400 rads) with the control shows that in the former both acellular and cellular cementum now reach almost to the base of the tooth, covering parts which in normal expectation would have moved towards the mouth and would have been replaced by new tooth material formed deep to them (Figs. 5, 6).

In correlation with the apposition of cementum, the periodontal membrane becomes occupied by the collagenous principal fibres almost to the base of the tooth (Fig. 6), a further abnormality of position due to failure of eruption and diminished growth. At the same time the quantity of fibroblasts in the periodontal membrane is by 30 days after injection (4400–9700 rads according to site) far less than normal (Figs. 13, 14) and their nuclei may appear large, pale and foamy.

The cementoblasts associated with the acellular cementum are by this time much reduced in number but they show great resistance and are not completely destroyed except in conjunction with necrosis of adjacent tissue near the gum. In many areas there are eventually very thick layers of acellular cementum (which can be associated with arrested movement of the cheek teeth towards the mouth) and irregular spurs formed by union with isolated cementicles. Pyknotic and fragmented nuclei are found near the cementoblasts and among the fibroblasts of the periodontal membrane from 3 days after injection, perhaps predominantly in regions where mitoses are frequent.

Enamel organs. In the cheek teeth the deepest parts of the enamel organs show damage early, some pyknotic cells being found near the area of injury of the pre-odontoblasts at 3 and 9 days (1400 rads) after injection, while mitoses are still occurring in the same area of the organ. Mitoses continue in that part of the organ superficial to this point up to 30 days but, deep to it, degenerative changes soon occur, the cells losing their characteristic morphology by about 14 days (2500 rads, Fig. 8) and apparently ceasing to grow. The exudate separating the internal enamel epithelium from the pre-odontoblasts extends to the deep tip of the organ (Fig. 10). Gradually the whole organ shrinks to a small island which does not disappear even after 6 months (12,700 rads). No neoplastic changes have been observed in it. In the cheek teeth the formation of some enamel matrix continues for a few weeks and sometimes much longer in teeth at the extreme ends of the tooth row.

In the incisors the enamel organ is less severely affected and production of enamel matrix is maintained for several months. Hertwig's sheath on the lingual side of the tooth is more severely affected and appears to be destroyed in certain areas in some teeth 60 days or more after injection (Fig. 11).

Bone. In the young rabbit there is normally very active remodelling taking place continuously in the bone around and between the teeth. In the injected animals, apposition of alveolar bone is greatly retarded, so that by 30 days after injection almost none appears still to be occurring (6000–9000 rads, Figs. 13, 14) except a little at the level of the bases of the teeth. Some resorption continues at most sites

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where it normally occurs, so that the quantity of alveolar bone gradually diminishes. Ultimately the remnants may be seen to have empty lacunae.

At some time between 60 and 90 days after injection, however, a very vigorous growth of bone-forming tissue commences. It arises on the surface of the bone of the sockets and in the mandible more in relation to the deep than the superficial half of them at first (8000–9000 rads). It is a pleomorphic osteoblastic proliferation such as has been previously described (OWEN *et al.*, 1957; DOWNIE *et al.*, 1959) and often contains giant cells with bizarre mitotic figures. It invades the periodontal membrane and adjacent marrow spaces (Fig. 15).

In rabbits killed after 90 days or more, the cheek teeth were solid right down to their bases and the pulps were reduced to button-like structures (Fig. 16). In general, the more superficial interdental bone and much of the old alveolar bone lying more deeply was now dead with very many empty lacunae, and the gum and adjacent periodontal membrane were often ulcerated and necrotic. In animals killed after 130 and 180 days, the periodontal membrane in the region where the osteoblastic proliferation had been seen in earlier specimens was partly occupied by dysplastic bone, on the surface of which there was little activity and which narrowed the membrane so much that tooth and bone were almost in contact (Fig. 16). In other parts pleomorphic proliferation was still very active, and in some it was associated with the resorption of bone and to a lesser extent tooth substance and the formation of tissue which often appeared to be osteosarcoma. In the left mandible of rabbit 1077 killed after 180 days (Fig. 17) three such areas, apparently quite separate, were present: around the incisor, between first and second premolars, and between first and second molars where the accumulated dose was estimated to be 15,000–20,000 rads (Fig. 18). In rabbit 1098, also killed after 180 days, most of the bone around the cheek teeth of the left mandible had been replaced by a bone-forming tumour in which numerous mitoses could be found. This growth was associated with tooth resorption and compression of the dental pulp in some areas (Fig. 19); in others with the apposition of osteoid on the tooth surfaces. Similar conditions were found in the left maxilla, and in both jaws of the third of the animals killed after 180 days. The left mandible of the fourth rabbit killed after the same interval had two clinical tumours both being osteosarcoma.

Injected dose of ^{90}Sr, 100–200 μc/kg *at the age of* 5–8 *weeks* . Rabbits injected with 100 μc/kg showed in their mandibular teeth relatively small qualitative effects. In the pulp near the deep extension of the enamel organs, separation of the pulp cells from the internal enamel epithelium was evident at 14 days after injection; and this had increased by 30 days (700 rads) at which time the exudate had extended to the tip of the enamel organ with apparently some thinning out of nearby pulp cells. At 60 days and later these abnormalities were much less evident or not seen. Pyknotic and fragmented fibroblasts were seen in the periodontal membrane 9 days after injection and thereafter, but the cellularity of the membrane was not obviously reduced.

Deformed growth of teeth was found in several cases, but what appear to be the late results of similar deformed growth were also found in some control rabbits

as young as 16 months in one case. The appearance suggested that the movement of the teeth towards the mouth had been impeded, while growth of the teeth continued, leading to undue pressure on the soft growing ends of the teeth and on the bone beneath them. It is not known whether these growth disorders are rendered more probable, earlier, or more severe by injection of ^{90}Sr. For this reason the findings and those in three weanling rabbits which received 200 μc/kg ^{90}Sr and were killed 19 or 24 months later will not be discussed.

DISCUSSION

The derangement of growth of the dental tissues after injection of 600 μc/kg was no doubt due to a combination of local effects of radiation on them and of effects influencing the health of the animal and affecting the teeth secondarily such as the varying degrees of anaemia and leucopaenia that may occur (OWEN *et al.*, 1957; OWEN and VAUGHAN, 1959a, b). The cellular damage observed at the bases of the teeth in 3 days or less can perhaps be regarded as a local result of radiation. This occurred at a dose-rate of from 5 to 8 rads/hr and was seen when the accumulated dose was from 160 to 500 rads. It is evident that the differentiating odontoblasts and adjacent pulp cells are particularly susceptible, also the growing tip of the enamel organ. The combined local and general effects of radiation thereafter are much less damaging at first to pulp and enamel organ cells already differentiated than to those immature.

The early and selective damage to the cells apparently about to become differentiated as columnar odontoblasts did not in fact in the incisors prevent the further supply of odontoblasts necessary to the increase in length of the tooth, since these were produced by proliferation of pulp cells or odontoblasts already differentiated. This does not indicate that in normal development odontoblasts are not produced from the area here damaged, but since a striking feature of the incisors of the experimental group was their progressive reduction in width, it may indicate that a principal source of pulp cells was removed by the damage to this area. The older odontoblasts and the cementoblasts showed great powers of resistance.

That cellular cementum continued to form near the bases of the cheek teeth at a time when apposition of bone on the alveolar interdental plates had ceased may be partly explained, however, by the fact that the accumulated dose at 30 days after injection was twice as great at the alveolar crest as it was near the base of the tooth. No neoplasia was observed in any of the dental tissues.

The pleomorphic osteogenic proliferation which appeared on the surface of alveolar bone in the deeper half of the sockets between 2 and 3 months after injection arose in an area which had received an accumulated dose of from 7500 to 9000 rads. It is interesting to note that the more superficial part of the alveolar bone had already received an accumulated dose of more than 9000 rads 1 month after injection, but that no such proliferation had arisen there or did arise until much later, if at all. The dose-rate there was twice as great at 9 days as at the base of the teeth and nearly three times as great at 30 days.

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In one case the osteolytic and odontolytic changes seen at 6 months after injection were found in a part which received an accumulated dose of between 15,000 and 20,000 rads. They did not occur in any animals in the more superficial alveolar bone when this had received a similar accumulated dose at about 2 months, but did so in two out of three rabbits at 6 months when the accumulated dose in that area had been doubled. It seems that in areas where the dose-rate was very high proliferative reactions were not accelerated and may have been retarded.

It is impossible to say at what stage neoplastic changes began, since the status of early pleomorphic proliferations is uncertain, but osteosarcoma was thought to be present in the mandibles of all four animals examined 6 months after injection. In three of these the growth extended from the gum to the lower border of the mandible through areas which had received from 11,000 to 52,000 rads. In the other, in which the particular lesion was small (rabbit 1077, Fig. 17) and the diagnosis perhaps less certain, the accumulated dose can be more nearly estimated at about 20,000 rads with dose-rate of about 9 rads/hr at 1 day, rising a little and then declining to about 5 rads/hr at 180 days.

It is of interest to compare the picture of the relation of radiation dose to radiation damage in the mandible, with that seen elsewhere in the skeleton. In two previous experiments the pattern of dose and damage in the long bones following ^{90}Sr administration has been studied in detail (OWEN and VAUGHAN, 1959a; DOWNIE *et al.*, 1959). In rabbits fed small daily doses of ^{90}Sr, about 8·5 μc/day, from the age of 5–8 weeks, after about 6 months abnormal bone which appeared to be the site of osteogenic sarcoma was widespread along the endosteal surfaces of the tibia. These regions of the tibia had received an accumulated dose of about 20,000 rads with a dose-rate increasing from zero to about 4 rads/hr. This is comparable with the mandible where sites of osteosarcoma had received an accumulated dose of the same order with dose-rates varying from 9 to about 1 rad/hr.

In rabbits given a single injection, in two cases there were small but frank tumours in the tibia occurring at 180 days. The dose at the site of tumour origin was estimated to be about 10,000–15,000 rads. One example of an early proliferative change similar to that noted on the surface of the bone of the tooth sockets about 90 days after injection, was also found. The accumulated dose received by the area in which the lesion had arisen was estimated to be about 12,000 rads (MACPHERSON, personal communication). This is of the same order as that received at the site of similar lesions in the mandible.

In the case of the long bones the early proliferative changes, so suggestive of sarcoma, are noted in areas that are already sites of active bone growth, the upper metaphysis and endosteal surfaces. Here in the upper metaphysis, where the histological changes have been studied in detail, there is proliferation of the osteogenic connective tissue in the marrow spaces between the trabeculae. Similarly in the case of the mandible there appears to be a stimulation by the radiation of abnormal osteogenic proliferation on the surfaces of alveolar bone near the deeper half of the sockets. These are regions where apposition and resorption are normally occurring. Malignant change may then occur in this proliferating tissue.

To summarize, it would appear that the sites of osteogenic sarcoma in the mandible and in the long bones are similar, i.e. the osteogenic connective tissue associated with bone surfaces which were sites of active apposition or resorption at the time of injection. Furthermore, where it could be estimated, it was found that about the same dose accumulated over 6 months produced tumours at these sites in both the mandible and long bones.

Acknowledgements—The investigations described were carried out with the aid of a grant for technical assistance from the Medical Research Council to one of us (M.A.R.). The authors wish to express their thanks to Miss J. GARDNER and Mrs. S. GROVES for technical assistance and to Mr. J. E. HUTCHINSON for histological preparations and photomicrographs.

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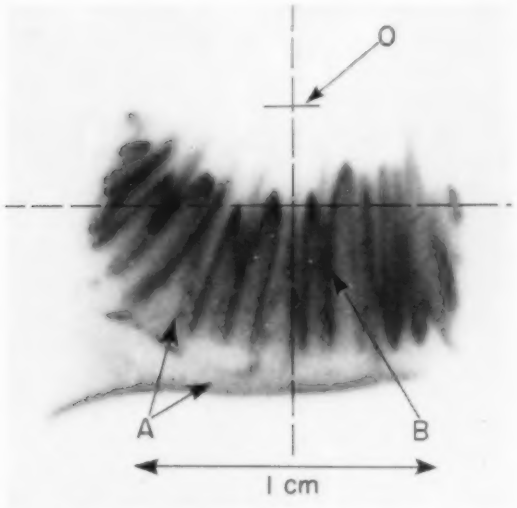

FIG. 1. Autoradiograph of mid-vertical plane through the cheek teeth of a rabbit given 600 μc of ^{90}Sr/kg and killed 24 hr after injection. Note the uptake of ^{90}Sr in alveolar bone, A, and in calcifying enamel and dentine which surrounds the tooth pulp chamber, B. The variation in dose-rate was measured along the dotted lines. The position of the occlusal surfaces is marked O.

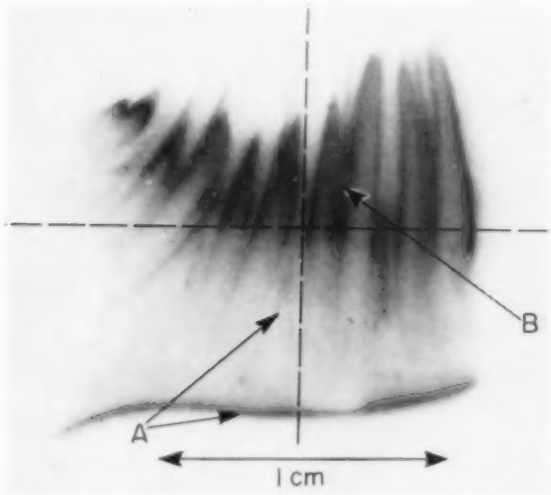

FIG. 2. Autoradiograph of mid-vertical plane through the cheek teeth of a rabbit given 600 μc of ^{90}Sr/kg and killed 30 days after injection. Note the retention of ^{90}Sr in alveolar bone, A, and in calcifying enamel and dentine, B. The latter deposit has moved towards the occlusal surfaces (compare with Fig. 1). The variation in dose-rate was measured along the dotted lines.

PLATE I

Fig. 5. Control 57 days after weaning. Base of molar. Haematoxylin and eosin. ×48.

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Fig. 6. (1056) Weanling rabbit which received 1000 μc/kg and was killed 30 days later. The more recently differentiated odontoblasts are abnormal and in relation to them the dentine contains included cells and capillaries. The cellular cementum at top left is almost in contact with the enamel organ at bottom left. Haematoxylin and eosin. ×48.

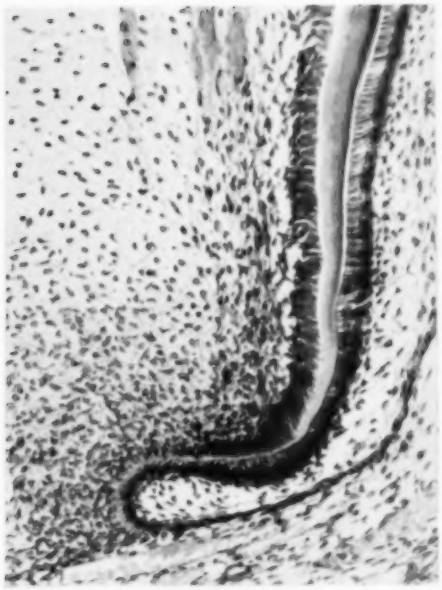

FIG. 7. (1035) Tip of enamel organ. Control as Fig. 1. Haematoxylin and eosin. ×160.

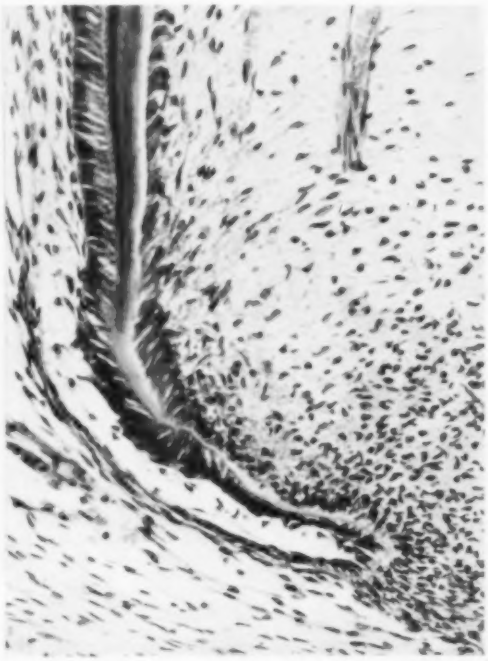

FIG. 8. (1136) Tip of enamel organ 14 days after injection of 600 μc/kg. Accumulated dose 2500 rads. Haematoxylin and eosin. ×180.

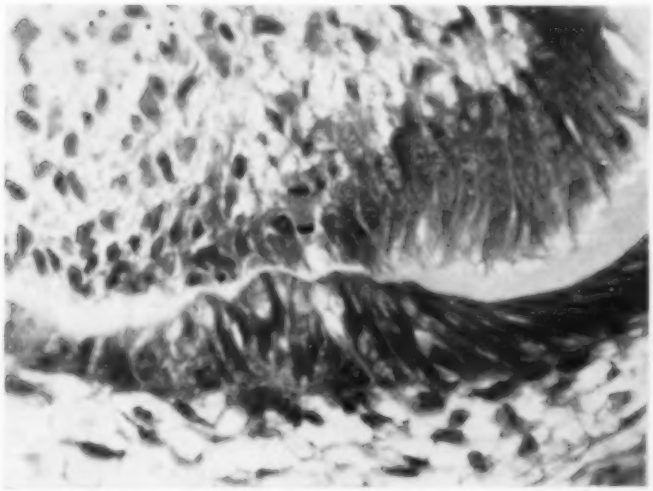

FIG. 9. (1053) Differentiating odontoblasts are separated from enamel organ by exudate (left centre) and some are pyknotic 3 days after injection of 1000 μc/kg. Recent dentine matrix is seen on right. Haematoxylin and eosin. ×450.

PLATE 3

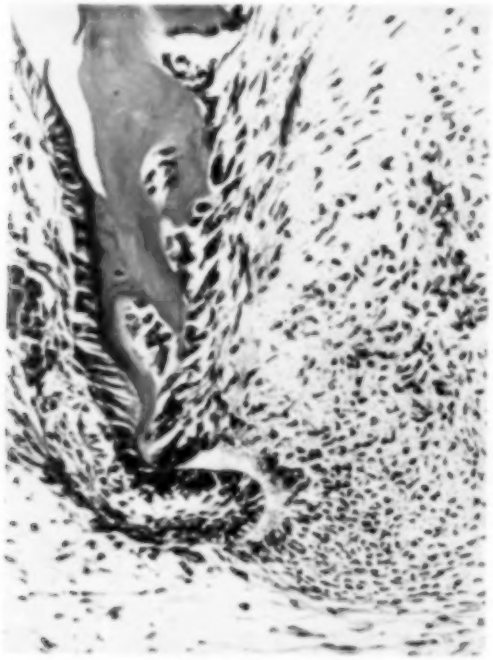

Fig. 10. (1140) 60 days after injection of 600 μc/kg. Enamel organ and recent dentine. Haematoxylin and eosin. ×180.

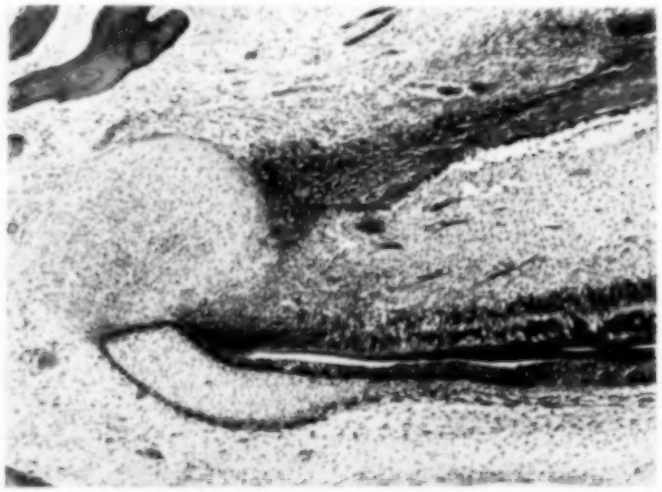

Fig. 11. (1140) Incisor 60 days after injection of 600 μc/kg, showing irregular dentine, segregation of immature pulp, and disappearance of Hertwig's sheath. Haematoxylin and eosin. ×36.

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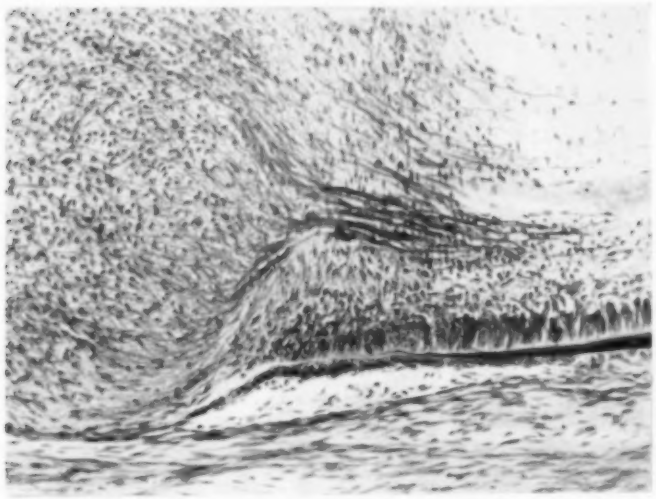

FIG. 12. (1151) Incisor 90 days after injection of 600 μc/kg, showing thick collagen fibre bundles. Van Gieson. ×108.

FIG. 13. Control, 57 days after weaning. Interdental plate of alveolar bone showing normal remodelling: resorption on left and apposition on right. Haematoxylin and eosin. ×80.

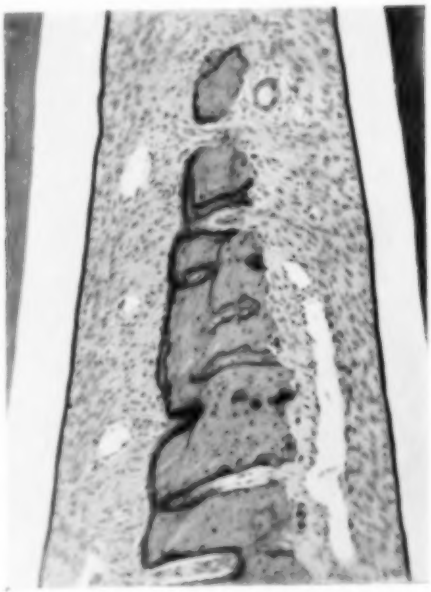

Fig. 14. (1137) Weanling injected with 600 μc/kg and killed after 30 days. Resorption continued but apposition had ceased. The periodontal membrane is less cellular. Accumulated dose 9700 rads. Haematoxylin and eosin. ×72.

Fig. 15. (1077) Weanling injected with 600 μc/kg and killed 6 months later. Osteoblastic proliferation on surface of wall of socket. Haematoxylin and eosin. ×80.

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FIG. 16. (1077) Weanling injected with 600 μc/kg and killed 6 months later. Teeth are solid and pulps greatly reduced. The periodontal membrane is partly occupied by dysplastic bone which at top left is almost in contact with the tooth. Haematoxylin and eosin. ×38.

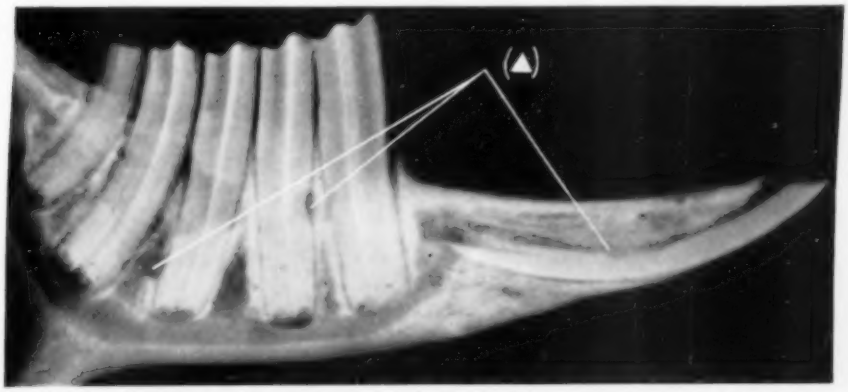

FIG. 17. (1077) Radiograph. Resorption of teeth has occurred at sites indicated.

Fig. 18. (1077) From mandible of rabbit injected as weanling with 600 μc/kg and killed 6 months later. One of three tumours which developed beside the teeth is shown at middle left. Haematoxylin and eosin. ×22.

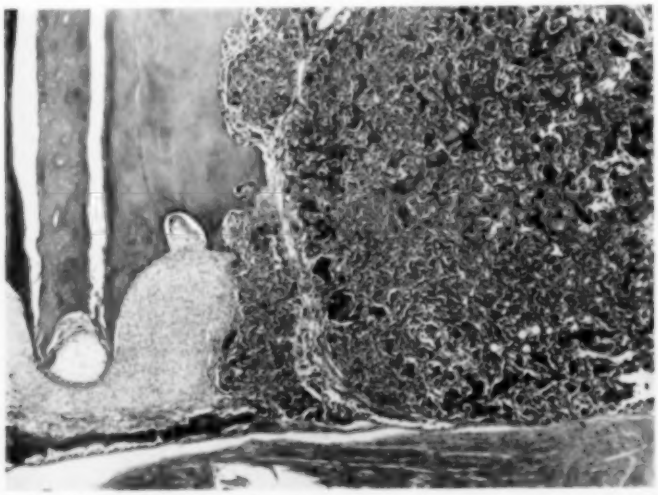

Fig. 19. (1098) Resorption of molar tooth and bone and compression of dental pulp by mandibular osteosarcoma in another rabbit killed 6 months after injection of 600 μc/kg. Haematoxylin and eosin. ×22.

Plate 8

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STUDIES ON THE INHERITANCE OF DENTAL CARIES IN THE HARVARD STRAINS OF CARIES-SUSCEPTIBLE AND CARIES-RESISTANT RATS

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Abstract—A crossbreeding experiment was conducted with the Harvard caries-susceptible and caries-resistant strains to determine the relative influence of the father, the natural mother and the nursing mother upon the caries activity of the offspring. The same adults of the two strains with appropriate matings were used for the production of pre-experimental and post-experimental purebred controls and for the production of crossbred offspring. A comparison was made of the dental caries experiences of 103 purebred caries-resistant rats, 83 purebred caries-susceptible rats, 262 representatives of the cross of susceptible males by resistant females and 178 representatives of the cross of resistant males by susceptible females. The individual caries experiences within the crossbred groups covered a very broad and continuous spectrum from very high caries-susceptibility to very high caries-resistance. The later in the experimental period that grossly visible carious lesions developed, the fewer lesions were initiated and the less rapidly they progressed. The average dental caries experiences of the rats from both crosses were very similar to each other. The average dental caries experiences of the two populations of crossbred rats were significantly higher than for purebred rats of the caries-resistant strain and significantly lower than for purebred rats of the caries-susceptible strain. The male and female parent exerted about equal influences upon the caries activity of the offspring, while the nursing mother had relatively little, if any, effect.

INTRODUCTION

THE PREDICTABILITY with which the phenotypic manifestations of the inherited traits of caries-susceptibility and caries-resistance were displayed in representatives of the Harvard strains despite vigorous attempts to alter the accessibility to oral micro-organisms has been described in an earlier paper (SHAW and GRIFFITHS, 1960). Maintenance of caries-susceptible offspring from within 24 hr after birth until weaning with caries-resistant foster mothers did not reduce the expected caries activity of the offspring. Likewise, the nursing of newborn caries-resistant babies by caries-susceptible mothers, or the maintenance of caries-resistant weanlings with caries-susceptible weanlings or with caries-susceptible adults which had numerous gross carious lesions throughout a complete caries assay period of 250 days did not result in significantly increased caries activity among the caries-resistant offspring.

The present experiment was undertaken to evaluate in greater detail the inter-relationship between the transmissibility of oral micro-organisms and the inheritance of caries-susceptibility and caries-resistance in the Harvard strains. Crossbreeding between the two strains was the primary procedure used. In addition, the fostering of offspring by females of the opposite strain to the natural mother was employed

in some groups. Thus the subjects could have various combinations of the three possible contacts with a given strain: through the father, through the natural mother *in utero*, or through a foster mother during lactation.

EXPERIMENTAL METHOD

The design of this experiment is presented in Table 1. The procedures involved made possible an evaluation of the influence of the two possible crossbred matings between the two Harvard strains: caries-resistant males with caries-susceptible females and caries-susceptible males with caries-resistant females. The influence of nursing the crossbred offspring by females which were of the opposite strain to their natural mother was also evaluated.

All females in this experiment were bred initially to provide one litter sired by males of their own strain. The offspring of these matings served as the pre-experimental control subjects to provide a baseline of the respective degree of caries activity for purebreds of each strain under current experimental conditions. After a normal post-weaning rest period, a second litter by the same female was sired by a male of the opposite strain. In order to build up adequate numbers in each crossbred group, most of the females had another litter by a sire of the opposite strain. Finally, the females bore an additional litter to a male of their own strain to serve as the post-experimental controls.

The pattern of fostering the offspring of one female by a female of the other strain within 24 hr after birth was conducted as in the previously reported experiment (SHAW and GRIFFITHS, 1960). Half of a litter born to a caries-susceptible female and sired by a caries-resistant male was transferred to a caries-resistant female who had just delivered a litter sired by a caries-susceptible male. At the same time half of the litter born to the caries-resistant female was transferred to the caries-susceptible female. Whenever crossbred litters were born at times when a suitable fostering transfer could not be made, the entire litters were reared by their natural mothers. None of the litters was reduced in size. Since the effect of fostering within each purebred strain had been tested adequately and the results reported previously, only the crossbred progeny were employed in the fostering procedure.

The offspring could be segregated into ten groups, as described in Table 1. The rats in Groups 1–5 were borne by caries-resistant females, while those in Groups 6–10 were borne by caries-susceptible females. The rats in Group 1 were the purebred pre-experimental caries-resistant controls reared by their own mothers. The rats in Group 5 were the purebred caries-resistant offspring from the last litters and served as post-experimental controls. The rats in Groups 6 and 10 compared exactly to those in Groups 1 and 5, respectively, with the exception that they were the pre-experimental and post-experimental purebred caries-susceptible controls, respectively. The rats in Groups 2, 3 and 4 were crossbreds of caries-susceptible males to caries-resistant females. Group 2 was composed of rats reared by their natural mothers; the rats in Group 3 were the littermates of the rats in Group 2 which had been fostered by caries-susceptible females. Thus the only contact of the offspring in Group 3 with the caries-resistant strain was during the gestation period. The rats

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TABLE 1. EXPERIMENTAL DESIGN OF CROSSBREEDING EXPERIMENT AND BODY WEIGHT INCREASES OF OFFSPRING

Group no.	Father	Mother	Foster mother	No. of of rats	Avg. increase in body weight at 100 days of age	
					♂	♀
1	Res.	Res.	None	40	299	189
2	Susc.	Res.	None	47	269	162
3	Susc.	Res.	Susc.	47	277	154
4	Susc.	Res.	None	168	287	177
5	Res.	Res.	None	63	292	176
6	Susc.	Susc.	None	35	268	147
7	Res.	Susc.	None	41	299	164
8	Res.	Susc.	Res.	51	326	176
9	Res.	Susc.	None	86	287	178
10	Susc.	Susc.	None	48	247	152

in Group 4 were identical genetically with Group 2 except that all the offspring in these litters had been reared by their natural caries-resistant mothers because no crossbred litter of a caries-resistant male to a caries-susceptible female had been available for fostering at the time of parturition. The rats in Groups 7, 8 and 9 were the products of caries-resistant males and caries-susceptible females. In Group 7, the rats were reared by their natural caries-susceptible mothers while their littermates in Group 8 were reared by foster mothers of the caries-resistant strain; thus the only dependence of the rats in the eighth group upon the caries-susceptible strain was through inheritance from their natural mother and during maintenance *in utero*. The rats in Group 9 were crossbreds of caries-resistant males with caries-susceptible females who reared all their own offspring because no suitable crossbred litter of the caries-susceptible male to caries-resistant female type was available for fostering at the time of birth.

Determination of an appropriate time to sacrifice the crossbred subjects in this experiment became a major problem that we had not encountered in experiments with purebreds of either strain. Preliminary oral inspection *in vivo* of the crossbred subjects indicated that they possessed the very wide range of caries-susceptibility that was typical of rats from many commercially available sources today and that was reminiscent of earlier years in our laboratory when concerted efforts were being made to establish caries-resistant and caries-susceptible strains.

Our usual procedure in the caries-susceptible strain of terminating an experiment at a single time around 80–100 days of age when the majority of the subjects had developed several grossly visible carious lesions was not practicable because the majority of the crossbred offspring were caries-free at this age. Likewise, the procedure of terminating experiments in which representatives of our caries-resistant

strain had been used at 250–260 days was impractical. This long experimental period could not be used because a considerable number of the crossbred offspring were so caries-active that their teeth would have been completely destroyed early in the period and a later evaluation of the caries process would have underestimated their caries activity. The best answer appeared to be sacrificing the crossbred rats at varying times based on the observed caries activity in each subject. Periodic caries examinations were conducted *in vivo* on the entire crossbred experimental population. No grossly visible carious lesions were observed before 60 days of age. Rats in which one or more grossly visible carious lesions appeared between 61 and 100 days of age were sacrificed on the 100th day. The rats in which one or more carious lesions became visible between 101 and 140 days of age were killed on the 140th day. Similarly, the other crossbred rats were killed on the 180th, 220th and 260th day of age. No comparable problem was involved with the pre- and post-experimental subjects of either purebred strain. The two control groups of the caries-susceptible strain were killed on the 100th day of age, while both groups of controls for the caries-resistant strain were killed on the 250th day of age.

The skulls were fixed in 95% alcohol and cleaned after 48 hr. The number of carious molars, the number of carious lesions, and the extent of carious lesions were determined by the method of SHAW *et al.* (1944). The designation on the rat skulls was coded so that their parentage, number of days on experiment and group identification were unknown to the evaluator until the entire experiment had been completed.

RESULTS

The results of the crossbreeding experiment are presented in Table 1 and Fig. 1. The cross between Harvard caries-susceptible males and Harvard caries-resistant females and the cross between Harvard caries-resistant males and Harvard caries-susceptible females both provided ample numbers of offspring for each experimental group. In view of the several intervals at which experimental subjects were sacrificed after the development of grossly visible carious lesions, the average gain in body weight for all animals in each group is presented at 100 days of age. At this stage the purebred representatives of the caries-resistant strain tended to be appreciably larger than the purebred representatives of the caries-susceptible strain. This difference in body weight is a characteristic of the two strains and continues throughout adult life. From Table 1 it can be seen that the average gain in body weight was very comparable for all crossbred groups with the exception of Group 8, where the males had attained greater increases in average body weights than in the other groups. In general there was a tendency for the average weights of the representatives of both sexes in the crossbred groups to be above the average weights for groups of the same sex among the purebred caries-susceptibles.

The data for dental caries experience among the ten groups of rats in this experiment are presented in the form of bar graphs in Fig. 1. The data for the purebred caries-resistant rats, pre-experimental controls in Group 1 and post-experimental controls in Group 5 indicate the generally high resistance to dental caries among

the representatives of this strain. For example, in the 40 rats in Group 1, only 7 per cent had grossly detectable carious lesions at 250 days of age and 78 per cent of the 40 rats were found to be completely free of minute or gross lesions in a detailed examination of the teeth. The average dental caries experience for the 40 rats in this group was 0·7±0·2 for the number of carious molars, 0·8±0·2 for the number of

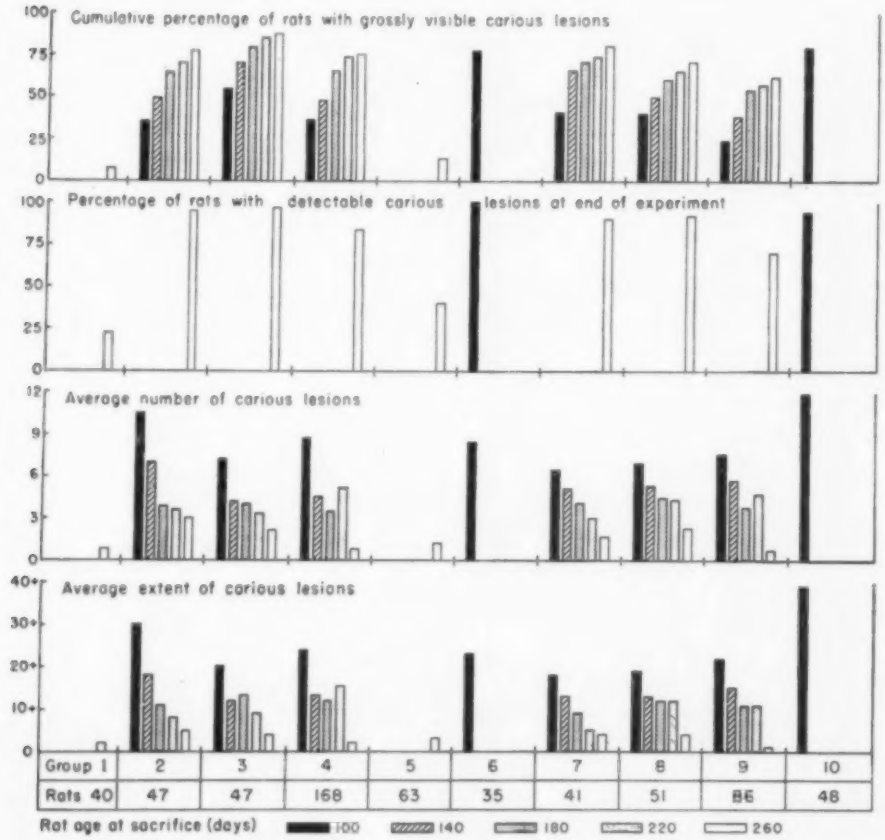
Cumulative percentage of rats with grossly visible carious lesions
Percentage of rats with detectable carious lesions at end of experiment
Average number of carious lesions
Average extent of carious lesions
Group 1 2 3 4 5 6 7 8 9 10
Rats 40 47 47 168 63 35 41 51 86 48
Rat age at sacrifice (days) 100 140 180 220 260

FIG. 1. The average dental caries experience of rats used to study the influence of crossbreeding the Harvard caries-resistant and caries-susceptible strains.

carious lesions and 1·7+±0·6+ for the extent of carious lesions at 250 days of age. Similar results were observed for the 63 rats in Group 5. Although there are minor differences in dental caries experience between the rats in Groups 1 and 5, there is no statistically significant indication that the caries-resistance of these later offspring had been increased as a result of the intervening experimental circumstances imposed upon the breeding animals, both males and females of the caries-resistant strain having been caged with caries-susceptible animals for prolonged periods.

In contrast to the high caries-resistance in Groups 1 and 5, the data for the purebred representatives of the caries-susceptible strain, the pre-experimental controls in Group 6 and the post-experimental controls in Group 10, indicate the generally high caries-susceptibility of these rats under the circumstances in this experiment. For example, 77 per cent of the 35 rats in Group 6 had grossly visible carious lesions at 100 days of age and 100 per cent had one or more lesions of all sizes. The average caries experience for this group can be expressed as $5{\cdot}0 \pm 0{\cdot}3$ for the number of carious molars, $8{\cdot}3 \pm 0{\cdot}8$ for the number of carious lesions and $22{\cdot}5 + \pm 2{\cdot}9 +$ for the extent of carious lesions. Similar results were observed for the rats in the later purebred litters in Group 10 which were born to the same parents. The actual extent of carious lesions for the latter group was significantly higher than for their older brothers and sisters in Group 6. This increase in caries activity is usually not seen in later litters of the same parentage, but serves in this case to indicate that none of the intervening experimental circumstances had reduced the ability of the susceptible male and female rats to pass on high caries-susceptibility to their offspring. There is no presently available explanation why this significant increase in caries activity should have occurred in view of the fact that there had been no known change in experimental circumstances.

The average data for the crossbred offspring of caries-susceptible males by caries-resistant females in Groups 2, 3 and 4 and for crossbred offspring of caries-resistant males by caries-susceptible females in Groups 7, 8 and 9 fall between the extremes for the purebred caries-resistant offspring at one end of the spectrum and for the purebred caries-susceptible offspring at the other end of the spectrum. However, in each of these six groups of crossbred offspring, substantial percentages (24–55 per cent) of the rats had grossly visible carious lesions at 100 days of age and a substantial percentage (13–41 per cent) had no grossly visible lesions even after 260 days of age. Smaller percentages of rats in each group developed grossly visible carious lesions during each of the three intermediate time intervals. In each of the six groups, small percentages (4–30 per cent) of the rats had no evidence of any carious lesions upon minute examination after sacrifice at 260 days of age. When judged on the basis of the time of detection of the grossly visible carious lesions, and the number and the size of the lesions, the rats killed at 100 days of age in the six crossbred groups were phenotypically as caries-susceptible as purebreds of the caries-susceptible strain. In contrast, those rats which had no detectable carious lesions when killed at 260 days of age were phenotypically as caries-resistant as purebred rats of the caries-resistant strain.

An interesting trend in caries activity that was dependent on age was observed among the crossbred rats. The rats which were killed at 100 days of age gave evidence of being highly caries-susceptible because the grossly visible carious lesions had developed earlier than among their littermates; in addition, their average number of carious lesions and their average extent of carious lesions were invariably higher than the comparable averages for their more caries-resistant littermates killed at any later stage. This decreasing caries activity with respect to number of carious lesions and extent of carious lesions was in general lower for each advancing age group

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and reached a minimum among rats killed at 260 days of age. For example, in Group 2, the 17 rats killed at 100 days of age had an average of 10·4 carious lesions, the 6 rats killed at 140 days had an average of 7·0 carious lesions, the 7 rats killed at 180 days had an average of 3·9 carious lesions, the 3 rats killed at 200 days had an average of 3·7 carious lesions and the 14 rats killed at 260 days of age had an average of 2·6 carious lesions. The comparable values for the average extent of carious lesions were 29·8+, 17·8+, 10·7+, 8·3+ and 4·9+, respectively.

An even more precise way to express this trend in its dependence upon time was to determine the average number of carious lesions and the average extent of carious lesions for each 40-day interval that the rats were on experiment. Since the rats killed at 100 days of age had been on experiment from 21 days of age, their experimental duration would be two 40-day intervals, whereas those rats killed at 140, 180, 220 and 260 days of age would be three, four, five and six 40-day intervals, respectively. Therefore the above data for carious lesions and for extent of carious lesions for the five subgroups of rats in Group 2 should be divided by 2, 3, 4, 5 and 6. On this basis, the average number of carious lesions per 40-day experimental interval for the rats in Group 2 killed at 100, 140, 180, 220 and 260 days of age would be 5·2, 2·3, 1·0, 0·7 and 0·4, respectively. Similarly, the average extent of carious lesions per 40-day experimental period for the same rats would be 14·9+, 5·9+, 2·7+, 1·7+ and 0·8+, respectively. This trend of decreasing caries activity with increasing age is statistically highly significant at the 1 per cent level for the number of carious lesions and for the extent of carious lesions. Under these circumstances, high caries-susceptibility in these populations expresses itself not only in the earlier appearance of lesions, but in higher numbers of rapidly progressing carious lesions.

In addition to the above general trends, the data in Fig. 1 needs to be examined to determine the relative influences that the father, the natural mother and the nursing mother have upon the phenotypic demonstration of caries activity. The rats in Groups 2 and 4 were comparable to each other in parentage, with caries-susceptible fathers and caries-resistant mothers; in addition, they were nursed by their natural mothers. The rats in the two groups differed only by reason of the probably inconsequential fact that the mothers of the rats in Group 2 also nursed some fostered rats from the opposite cross of caries-resistant males by caries-susceptible females. The cumulative percentages of rats with grossly visible carious lesions at the five 40-day intervals were almost identical for the two groups. The percentage of rats with any detectable carious lesions at the end of the experiment was higher in Group 2 than in Group 4. Likewise, there was a trend toward a lower dental caries experience among the rats in Group 4 but this difference between the two groups was not statistically significant.

When the data for the crossbred rats in Groups 2 and 4 were compared with the data for the purebred caries-resistant Groups 1 and 5 and for the purebred caries-susceptible Groups 6 and 10, the dental caries-susceptibility of the crossbreds was seen to be increased from the purebred resistants and decreased from the purebred susceptibles by highly significant amounts in both cases. In other words, when the genetic tendency to high caries-susceptibility was represented only in the male parent, a highly significant influence was exerted on the average dental caries experience

among crossbred offspring. Likewise, the caries-resistant mother during the total period of gestation and lactation has a highly significant influence upon the average dental caries experience of the crossbred offspring.

The rats in Groups 7 and 9 were comparable to each other in parentage, with caries-resistant fathers and caries-susceptible mothers; in addition, they were nursed by their natural mothers. The rats in the two groups differed only by reason of the use of the females in Group 7 to nurse offspring from the opposite cross. The cumulative percentages of rats with grossly visible carious lesions at the end of the five intervals tended to be lower in Group 9 than in Group 7 by differences which are statistically significant at the 5 per cent level. Likewise, the percentage of rats with any detectable carious lesions at the end of the experiment was appreciably higher in Group 7 than in Group 9. However, the rats in Group 9 killed at the end of each of these periods had average dental caries experiences that were in general as high or higher than comparable rats in Group 7.

When the data for the crossbred rats in Groups 7 and 9 were compared with the data for the purebred caries-susceptibles in Groups 6 and 10 and for the purebred caries-resistants in Groups 1 and 5, the dental caries-susceptibility of the crossbreds was greater by a highly significant amount than that of the caries-resistant purebreds and was lower by a highly significant amount than that of the caries-susceptible purebreds. As in the previous comparison between Groups 2 and 4, both the caries-resistant father and the caries-susceptible mother during the total period of pregnancy and lactation had had a highly significant influence on the average dental caries experience of the crossbred offspring.

In order to determine the influence of the strain of the nursing mother upon the dental caries experience of the crossbred offspring, the rats in Group 2 should be compared with those in Group 3 and the rats in Group 7 should be compared with those in Group 8. The rats in Groups 2 and 3 were littermates, with caries-susceptible fathers and caries-resistant mothers; the rats in Group 2 were nursed by their natural mother, while those in Group 3 were nursed by caries-susceptible foster mothers. Thus the only relationship of the rats in Group 3 with the caries-resistant strain was by their mother during gestation. The cumulative percentages of rats with grossly visible carious lesions during the five intervals were significantly higher in Group 3 than in Group 2. This observation suggested that the caries-susceptible nursing mother may have been responsible for a slightly earlier onset of carious lesions. However, when the total percentage of rats developing any detectable carious lesions during the experiment is considered, there was no difference between the two groups of rats. It is noteworthy that the average dental caries experience of the rats killed at each time interval except the third were lower in Group 3 than in Group 2. This trend toward a lower caries activity among the rats in Group 3 was true for both the average number of carious lesions and the average extent of carious lesions. Thus these two groups of observations tend to cancel each other out, indicating that the caries-susceptible nursing mothers may have caused a slightly earlier onset of lesions but clearly had no effect on the average dental caries experience of the offspring. The rats in Groups 7 and 8 again are littermates, but this time with caries-resistant

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fathers and caries-susceptible mothers. The rats in Group 8 were nursed by caries-resistant mothers, while those in Group 7 were nursed by their natural mothers. Thus the only contact of the rats in Group 8 with the caries-susceptible strain was with the mother during gestation. The cumulative percentages of rats with grossly visible carious lesions at the end of the five intervals and the average dental caries experiences of the rats killed at each interval were almost exactly identical for Groups 7 and 8. Likewise, the percentages of rats in the two groups which developed any detectable carious lesions during the experiment were comparable. This observation again provided a clue that the offspring have little or no need for direct contact with a caries-susceptible subject during the lactation period for a full expression of their inherited caries-susceptibility to be displayed.

In summary, the data from these six groups of crossbred rats indicate that the caries-susceptibility in our strains of rats is about equally dependent upon the inheritance from the father and from the mother and that their caries activity is influenced relatively little, if at all, by the caries-susceptibility or the caries-resistance of the nursing mother. In addition, the average dental caries experiences of the representatives of the two separate crosses, susceptible male by resistant female and resistant male by susceptible female, were indistinguishable from each other.

Considerable variations in caries activity were observed within a high percentage of the crossbred litters. It was common for litters to contain one or more members that exhibited a very high caries activity, several members with intermediate levels of caries activity and one or more members with a very low caries activity or freedom from caries. The average dental caries experiences of most litters were in the middle third of the spectrum. However, the average dental caries experiences of some litters were at the more caries-susceptible end of the spectrum, while the averages for other litters were at the more caries-resistant end of the spectrum. In the search for a possible explanation of this distribution, the caries activities of the purebred semi-siblings of the more caries-susceptible crossbred and the more caries-resistant crossbred litters were compared. At least one of the parents of each crossbred litter which exhibited the more resistant characteristics on the average had also contributed litters to the purebred pre- and post-experimental control groups that were more resistant than the average for their strain. Likewise, at least one of the parents of each crossbred litter which exhibited the more susceptible characteristics on the average had also contributed litters to the purebred pre- and post-experimental control groups that were more susceptible than the average for their strain. The obvious implications of these observations are that the two strains are not homogeneous and that the inheritance of proneness to experimental dental caries is complex.

DISCUSSION

The development of strains of rats with predictable susceptibility to dental caries under standardized conditions has been undertaken along genetic lines in the laboratories at Michigan State University and the Harvard School of Dental Medicine. The offspring of these strains displayed unmistakable phenotypic evidence of their

genetic inheritance when fed an appropriate cariogenic diet. On this basis representatives of the caries-susceptible strains could be readily distinguished from representatives of the caries-resistant strains.

Evidence has become available recently that dental caries can be considered to be a transmissible disease. KEYES (1960) has demonstrated that rodents with an inherent capacity to develop carious lesions would remain essentially free of active lesions unless their oral cavities became contaminated directly with microbial flora from animals of the same species with actively progressing lesions. In tests with the Hunt–Hoppert and the Harvard caries-resistant strains of rats, ROSEN, HOPPERT and HUNT (1960) and SHAW and GRIFFITHS (1960) have demonstrated that intimate contact with rats of the comparable caries-susceptible strains had little or no effect on the caries activity of the resistant rats. Conversely, segregation of the caries-susceptible rats of these strains to permit the minimum contact with adult rats of the same strains with active carious lesions did not result in decreased caries activity. These latter studies strongly suggested that the phenotypic selection and breeding procedures followed in the development of these lines had resulted in strains that could be termed accurately inherently caries-susceptible and caries-resistant. This conclusion does not exclude or disprove Keyes' concept of transmissibility. However, it seems evident from these studies that the necessary cariogenic flora can be readily established in the oral cavities of inherently caries-susceptible rats. On the other hand, in inherently caries-resistant rats the cariogenic flora either cannot be established readily, or if it can be established, cannot cause any appreciable increase in caries activity.

Additional support has been provided in the cross-breeding experiment for the concept that inherited differences determined caries activity when other factors of diet and environment were constant. The broad and continuous spectrum of caries activity from very high caries-susceptibility to very high caries-resistance observed among the offspring of the crossbred matings provided phenotypic evidence of their varied constitutional inheritance. The variation in caries activity within individual litters was the most explicit manifestation of caries inheritance in the midst of an almost ideally constant environment. Here all members of a litter have had as nearly constant uterine, nursing and post-weaning environments as could be designed.

Under these experimental circumstances the average dental caries experience for the offspring of the cross of susceptible males on resistant females was almost identical to the average for the offspring of the cross of resistant males on susceptible females. In addition, the average genetic contributions of the male parent and of the female parent were apparently of about equal importance. While the nursing mother may have had slight importance in determining the time of onset of carious lesions, this contribution was clearly very minor compared to the genetic influence of the father and of the natural mother.

The presence of this large group of crossbred progeny with their broad continuum of caries activity and comparable dietary background made possible studies of various criteria for judging the probable caries-susceptibility of a given subject. The major criterion used in our laboratory as an index of caries-susceptibility has been the time

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at which grossly visible carious lesions can be detected during an *in vivo* oral examination. This criterion has enabled us to determine with accuracy the time at which an experimental population should be sacrificed in order to make careful comparisons of the degree to which tooth substance has been involved in the carious process. In addition, in these crossbred progeny it was found that the early onset of lesions was accompanied by a greater rate of incidence of lesions and by much more rapid progress of the lesions. As the first evidence of grossly visible lesions was delayed in time, the number of lesions and the rate of progress of the lesions decreased precipitously.

In view of the lack of homozygosity in the two strains, no plans were made to breed any of the crossbred offspring for studies in the second filial generation. The primary purpose of the experiment has been achieved in the testing of the validity of the hypothesis that the phenotypic evidences of caries activity under the standardized conditions of the experiment were true measures of the inherited tendencies toward various levels of caries-susceptibility.

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EFFECTS OF SOME PHOSPHATES IN A SHORT-PERIOD RAT CARIES EXPERIMENT

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Abstract—The effect of supplementation of sodium pyrophosphate, sodium metaphosphate, disodium phosphate and disodium glycerophosphate to a high-carbohydrate low-fat diet (Stephan 580) and of administration of phosphoric acid in drinking water on caries incidence was studied in rats for 20 days. Meta-, pyro- and orthophosphates inhibited caries diagnosed microscopically. Phosphoric acid did not inhibit caries but caused erosions in the lingual surfaces of the molars. In addition, the effect *in vitro* of phosphates was tested in an acid gelatine assay and by measuring the inhibition of fall in pH in incubated saliva–sucrose mixtures.

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IN 1931, KLEIN and MCCOLLUM for the first time presented evidence pointing to the importance of the level of phosphorus in the diet as a factor in producing caries susceptibility and caries resistance in rats. Some years later, SOBEL and HANOK (1948) published results indicating that the calcium:phosphorus ratio of a diet may influence the inorganic composition (i.e. phosphate:carbonate ratio) of rats' incisors. In subsequent experiments, SOBEL (1952, 1955, 1960) could show a relationship between the Ca:P ratio of a diet and the caries-susceptibility of the molars formed during administration of the diet. WYNN *et al.* (1956, 1957) and HALDI *et al.* (1959) concluded from a number of experiments in albino rats that the cariogenicity of a diet must be due to the actual phosphorus and calcium content and not to the Ca:P ratio. The same conclusion was arrived at in a recent paper by MCCLURE and MCCANN (1960). The role played by small amounts of naturally occurring organic phosphorus as a solubility-reducing and caries-inhibiting factor, e.g. in refined "white" and unrefined "brown" flour, is a problem still under *in vitro* investigation (OSBORN, 1941; JENKINS *et al.*, 1959).

However, animal caries experiments carried out with diets supplemented by various soluble and insoluble inorganic and organic phosphates have shed some light on the possibilities of caries control.

STRALFORS (1954), in his early experiments on hamsters, tested the soluble alkaline tertiary sodium phosphate. In subsequent tests *in vitro*, hamster experiments and clinical trials STRALFORS (1956, 1959) studied diets supplemented with the less soluble calcium phosphates, observing a reduction of the enamel dissolution rate *in vitro*, and good caries reduction in hamsters. According to STRALFORS' theory, calcium phosphate is to be preferred to sodium phosphate, as it avoids the risk of an unbalanced Ca:P ratio. Calcium phosphate incorporated into the plaque will go into

solution when the pH falls. The temporary and local increase of the calcium as well as of the phosphate concentration might reduce the dissolution of these ions from the enamel.

Recent observations stress the importance of the amounts of soluble phosphorus in diets actually available to yield a caries inhibiting effect. McCLURE (1958) and McCLURE and MULLER (1959a, b) found a caries-inhibiting effect of the soluble dibasic sodium phosphate and also of dibasic calcium phosphate when solubilized by addition of sodium chloride. These findings are confirmed by VAN REEN and GLASSFORD (1959) and indirectly as well as directly by BARNARD and JOHANSEN (1958a, b) who found dibasic calcium phosphate ineffective in reducing caries when administered in a diet low in NaCl and other inorganic salts (Stephan diet 580) but effective, nevertheless, in diets containing 1 and 5 per cent sodium chloride. NIZEL and HARRIS (1955, 1960) and their collaborators (NIZEL *et al.*, 1958; HARRIS and NIZEL, 1959; NIZEL, HARRIS and PARKER, 1960) observed a significant decrease of caries in hamsters maintained on cariogenic diets containing food ashes, metaphosphoric acid and monobasic potassium phosphate.

The experiment presented in this paper was designed to test some soluble phosphates in a short-period rat caries assay.

Experimental periods as short as 10–25 days in highly caries-susceptible Osborne–Mendel rats were used by the authors for several reasons (MÜHLEMANN *et al.*, 1956; MARTHALER, MÜHLEMANN and KÖNIG, 1957; KÖNIG, MARTHALER and MÜHLEMANN, 1958a, b). Influenced by BIBBY's (1954) historical reminiscence: "My studies of dental caries are based upon Dr. PICKERILL's statement that if dental decay is to be prevented, it must be prevented at the enamel surface", it seemed necessary to study initial lesions in experimental dental caries rather than advanced cavities. It is of limited practical interest to look for preventive measures resulting in destruction of only one fourth of the molar crowns compared with destruction of the total crowns in the control group. It may be assumed that the carious process becomes irreversible very early, at a stage of development when it has reached the dentine and is spreading along the dentine–enamel junction and undermining the enamel. This progress of the lesions beyond the reversible stage can be conveniently studied by microscopic examination of undecalcified serial sections of rat molars after cariogenic periods between 10 and 25 days long.

Moreover, short period caries studies offer the possibility of investigating lesion patterns in different strains, of discrimination between inhibition of initiation and progression, and of applying a variety of special experimental conditions. For instance comparing the local effect of inhibitors on erupted first and second with the systemic effect on calcifying third molars (MARTHALER *et al.*, 1957). For all these purposes sulcal lesions are sufficiently advanced after about 20 days on a highly cariogenic diet to assess microscopically differences in caries incidence between different treatment groups.

Even though fissure caries progresses, the "smooth surface" enamel generally remains intact in short period caries experiments. So it is possible to determine the enamel dissolution rate previous to sectioning the molars for microscopic caries

diagnosis. In most cases this is not a very conclusive and important finding, but if it has been altered in the experimental groups we may assume that the inhibitor applied has exerted some kind of local effect on the enamel surface. Considering the relations between inhibitory effects on dissolution of enamel and on caries incidence, it is necessary to be aware of the fact that solubility is determined on the easily accessible smooth surfaces, while lesions start usually in the fundus of the narrow sulci which almost always are obliterated by food impaction.

MATERIAL AND METHODS

Sixty-six weanling Osborne–Mendel rats were used to investigate the caries-inhibiting potentiality of the following phosphate compounds:

	Conc. per cent	No. of rats
(a) Sodium hexametaphosphate $(NaPO_3)_6$	2·63 (0·8 *P*)	11
(b) Sodium pyrophosphate $(Na_4P_2O_7.10\ H_2O)$	5·76 (0·8 *P*)	9
(c) Di-sodium glycerophosphate $C_3H_5(OH)_2OPO_3Na_2$	2·0 (0·29 *P*)	11
(d) Sodium orthophosphate, mono-H $Na_2HPO_4.12\ H_2O$	2·0 (0·17 *P*)	11
(e) Ortho-phosphoric acid (H_3PO_4)	0·1 (0·03 *P*)	10
(f) Water control group		14

Compounds (a)–(d) were added to the cariogenic diet, whereas phosphoric acid was given in the drinking water.

Littermate groups of six rats were assigned at random over the six treatments, irrespective of sex, and were housed in single metal cages with front and bottom wire mesh. For an experimental period of 20 days, they were given distilled water and diet 580 (sucrose 66 per cent, skim milk powder 32 per cent, whole dried liver 2 per cent) *ad libitum*. Weight gains were checked every fifth day. At the end of the experiment the lower jaws were carefully prepared, brushed with water, dried for 20 min at room temperature and covered with hard wax with exception of the molar crowns. Enamel surface solubility (KÖNIG *et al.*, 1958a) was then determined by exposing the uncovered molars to 12·5 ml pH 4 phthalate buffer under continuous shaking for 1 hr at a temperature of 37°C. Analyses of phosphorus were carried out according to the method of FISKE and SUBBAROW (1925) modified by LOHMANN and JENDRASSIK (1926).

The jaws were then freed of the wax, fixed in 10% formalin and sectioned serially with an abrasive disc microtome (JANSEN, 1950) in the mesio-distal plane of the teeth. The serially mounted sections were stained by the periodic acid–Schiff technique and examined microscopically for sulcal carious lesions (KÖNIG *et al.*, 1958b).

The lesions were scored as follows (with corresponding KEYES' (1958) nomenclature in parentheses):

A(E) = enamel lesion.
T(Ds) = involvement of dentine–enamel junction.
B(Dm) = moderate dentinal lesion.
C(Dx) = extensive dentinal lesion with undermining of cusps and beginning cavitation discernible macroscopically.

It is assumed that the lesions will develop progressively from stage A to T, B, C. Therefore, the scores given represent the total of lesion scores, A being the number of enamel lesions actually present plus the number of all more severe, T, B and C lesions. Details of the method have been reported previously (KÖNIG *et al.*, 1958b).

In order to obtain further knowledge of the mechanism whereby the effect of phosphate is produced, the animal experiments were supplemented by two *in vitro* tests.

(1) The ability of phosphates to inhibit acid formation in saliva–sucrose mixtures was studied (FOSDICK and KROB, 1960). 0·5 ml of saliva and 0·5 ml of RICKLES' (1953) indicator–sucrose solution were mixed with 0·1 ml of inhibitor solution in a test tube. A series of such test tubes with inhibitor solutions of varying concentrations was incubated at body temperature. Acid formation was checked colorimetrically each hour and finally after 4 hr measured with a Metrohm pH-meter.

(2) The ability of phosphates to inhibit artificial white spot formation (BARTHELD, 1958; MÜHLEMANN, 1960) was tested by a "gelatine decalcification assay" (MÖRCH, TORELL and HALS, 1957). Intact human teeth were incubated at 37°C for 6 days in a 2% gelatine solution acidulated with 1·0 M lactic acid to a pH of 4. The teeth were then inspected macroscopically for surface changes.

RESULTS

The effect of phosphates administered during the cariogenic period on (a) enamel solubility (removal of phosphorus) and (b) sulcal caries is represented in Fig. 1 and Table 1. Data are given for phosphorus removal in milligrams per 100 ml, and for sulcal caries in average numbers of lesions ± standard deviation.

Significant reductions of phosphorus removal were produced by sodium hexametaphosphate ($P_t < 0{\cdot}001$), sodium pyrophosphate ($P_t < 0{\cdot}01$) and orthophosphoric acid ($P_t < 0{\cdot}05$); the slight increase in removal of phosphorus caused by a supplement of di-sodium glycerophosphate to the diet was not significant ($0{\cdot}1 > P_t > 0{\cdot}05$).

The number of sulcal lesions (Table 1) indicated good protection against the caries attack by tetra-sodium pyrophosphate and by sodium hexametaphosphate. Initial (Score A and T) lesions were not effectively inhibited by sodium orthophosphate but the progression from initial to deeper dentinal lesions (Score B) was significantly depressed ($P_t < 0{\cdot}001$). Ortho-phosphoric acid did not inhibit the formation of sulcal carious lesions; on the other hand, erosive lesions on the lingual smooth surfaces of upper and lower molars were a consistent finding in this group. The eroded enamel and dentine surfaces were quite smooth (Fig. 2, Plate).

TABLE 1. EFFECT OF PHOSPHATES ON ENAMEL SOLUBILITY AND CARIES EXPERIENCE IN OSBORNE–MENDEL RATS

Enamel dissolution rate (mg P per 100 ml); average number ± standard deviation of Score A, T and B lesions; significance levels. Weights ± s.d. at the end of 20 day experimental period (age 45 days)

Treatment	No. rats	Removal of phosphorus (mg/100 ml)	Number of lesions score			Weight (g)
			A	T	B	
(a) Sodium metaphosphate	11	1·09 ± 0·13	11·18 ± 1·89	6·82 ± 2·18	1·09 (12 lesions in 5 rats)	93·7 ± 11·9
(b) Sodium pyrophosphate	9	1·30 ± 0·15	9·91 ± 2·17	2·45 ± 2·02	0·44 (4 lesions in 2 rats)	90·4 ± 15·6
(c) Di-Na-glycerophosphate	11	1·61 ± 0·13	13·36 ± 1·50	9·82 ± 2·82	4·91 ± 3·91	101·7 ± 17·5
(d) Sodium orthophosphate	11	1·56 ± 0·16	13·00 ± 1·84	8·73 ± 2·72	$0{\cdot}1 > P_t > 0{\cdot}05$ 2·73 ± 1·95 $P_t < 0{\cdot}001$	105·5 ± 17·7
(e) Ortho-phosphoric acid	10	1·30 ± 0·25	14·64 ± 1·36	10·54 ± 3·11	6·30 ± 4·03	104·6 ± 10·2
(f) Controls	14*	1·50 ± 0·16	14·27 ± 1·42	11·73 ± 3·07	7·54 ± 3·17	102·2 ± 16·1

* In the analysis of variance of lesion numbers only eleven rats were taken into account; these eleven control rats belonged to the eleven litters used for the two-way analysis of variance. The three missing values in the pyrophosphate and phosphoric acid groups were substituted according to SNEDECOR (*Statistical Methods*, 5th Ed., p. 310).

	a	b	c	d	e	f
a	—	***	*		**	***
b		—	***	***	***	***
c	**	***	—			
d	**	***		—		*
e	***	***			—	
f	***	***				—

Score A lesions

Score T lesions

* $P_t < 0{\cdot}05$
** $P_t < 0{\cdot}01$
*** $P_t < 0{\cdot}001$

Extensive dentinal (Score C) lesions were not frequent enough in this experiment to be treated statistically and were therefore added to the B-lesions. Nevertheless, the evaluation of the initial (Score T) and moderate dentinal (Score B) lesions was highly significant.

The phosphate effects on the sucrose–saliva mixtures are summarized in Table 2 and, in Fig. 3, they are graphically compared with some known inhibitors (sodium fluoride, stannous fluoride, iodoacetic acid and sodium penicillin G) tested under the same conditions. The initial pHs of phosphate-free control saliva–sucrose mixtures were between 7·0 and 7·5 (mean ± standard deviation = 7·22 ± 0·06; thirteen samples). The initial pHs in the phosphorus-containing saliva–sucrose mixtu es indicate the pH shifts due to the buffering capacity of the phosphate "inhibitors". As a criterion for the effect on acid formation, the molar concentrations of phosphates

preventing a pH drop below 5 within 4 hr were chosen. In this respect, sodium metaphosphate and pyrophosphate were superior to orthophosphate and glycerophosphate. This superiority was likewise observed in the inhibition of experimental caries. As a whole, the ability of the tested phosphates to inhibit a drop of the pH in the saliva–sucrose mixtures was definitely inferior to the inhibitory action of penicillin, iodoacetic acid and of the fluorides.

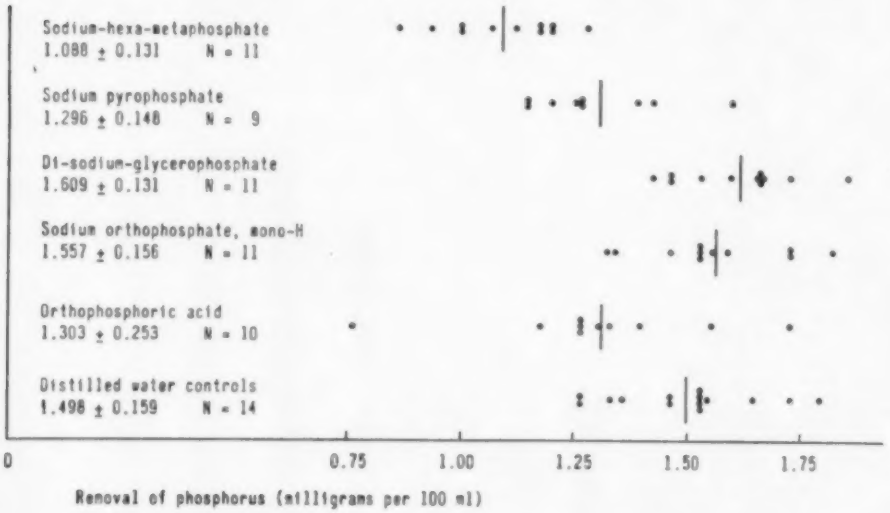

FIG. 1. *In vivo* effect of phosphates of enamel solubility in a rat caries experiment: removal of phosphorus from the crowns of the lower molars in acid buffer. Single values, averages ± standard deviation in milligrams per 100 ml.

TABLE 2. EFFECT OF SODIUM PHOSPHATES ON ACID FORMATION IN SALIVA–SUCROSE MIXTURES

Initial pH and pH after 4 hr of incubation at 37°C for series of samples at different concentrations. Italic type numbers: molar concentrations preventing drop of pH below 5 within 4 hr (cf. Fig. 3)

		Molar concentration											
		1·0	0·5	10^{-1}	$0{\cdot}5 \times 10^{-1}$	$0{\cdot}25 \times 10^{-1}$	10^{-2}	$0{\cdot}5 \times 10^{-2}$	$0{\cdot}25 \times 10^{-2}$	10^{-3}	$0{\cdot}5 \times 10^{-3}$	$0{\cdot}25 \times 10^{-3}$	10^{-4}
Metaphosphate	Initial			6·42	6·60	6·80	6·80	6·82	6·80	*7·03*	7·00	7·00	6·95
	Final			6·25	6·45	6·60	6·37	6·49	6·40	*5·75*	4·40	4·29	4·14
Pyrophosphate	Initial			9·38	9·33	9·26	8·84	*8·45*	7·78	7·20	7·10	7·00	6·90
	Final			9·49	9·26	9·10	8·10	*5·52*	4·55	4·32	4·25	4·24	4·14
Glycerophosphate	Initial	8·50	8·50	7·95	7·85	*7·70*	7·55	7·45					
	Final	8·50	8·35	6·22	5·64	*5·02*	4·77	4·54					
Orthophosphate	Initial	8·92	8·75	8·40	8·25	8·02	7·90	7·70					
	Final	8·90	8·81	7·92	6·70	5·48	*5·06*	4·72					
Controls	Initial	7·20	7·07										
	Final	4·08	4·14										

The inhibitory mechanism of iodoacetic acid and antibiotics is well known, but as far as phosphates are concerned it would be interesting to know whether the inhibition of fall in pH caused by them is due to their buffering capacities or to antibacterial properties.

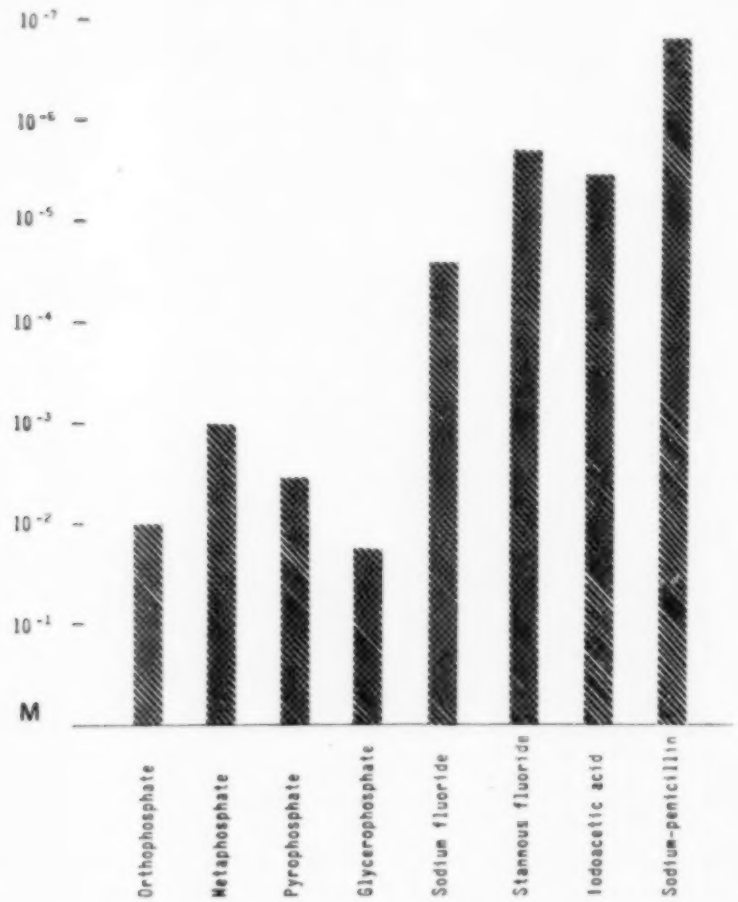

FIG. 3. Molar concentrations of phosphates, fluorides and antibacterial agents preventing drop of pH below 5 within 4 hr (cf. Table 2) in saliva–sucrose mixtures.

For discrimination between the two mechanisms, the following experiment was carried out. Eight pairs of test tubes with saliva–sucrose mixtures made of the same saliva samples were prepared. To four pairs phosphates (adjusted to the pH of the saliva by adding HCl or NaOH) were added to yield a concentration of: meta-

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phosphate, 10^{-3} M; pyrophosphate, $0{\cdot}5\times10^{-2}$ M; glycerophosphate, $0{\cdot}25\times10^{-1}$ M; and orthophosphate, 10^{-2} M, respectively. The concentrations chosen were those found effective just to inhibit a pH drop below 5 within 4 hr of incubation (see Table 2). The adjustment of the phosphate stock solutions to the pH of the saliva was done to get the same starting point for all the samples, the supplement of unadjusted pyrophosphate, for instance, causing a shift too far away from the growth optimum of streptococci. Together with these phosphate supplemented samples the four pairs of controls without phosphates were incubated for 4 hr. After incubation, the same amounts of phosphates as in the four corresponding pairs of samples supplemented with phosphates from the beginning of incubation, were added to the four pairs of control test tubes. If the prevention of pH drop was due to the buffering capacities of the phosphates only, their addition after incubation should give the same final pH reading as if they had been added before incubation.

TABLE 3. DROP OF pH IN EIGHT PAIRS OF SALIVA–SUCROSE MIXTURES TO WHICH DIFFERENT PHOSPHATES WERE ADDED BEFORE AND AFTER INCUBATION AT 37°C FOR 4 hr

(a) Initial pH, (b) final pH after incubation, (c) control pH after addition of phosphates. (i) added before incubation, (ii) added after incubation

		a	b	c
Metaphosphate (10^{-2} M)	(i)	7·3	6·6	
		7·3	6·7	
	(ii)	7·4	4·6	4·8
		7·4	4·6	4·8
Pyrophosphate ($0{\cdot}5\times10^{-2}$ M)	(i)	7·4	5·8	
		7·4	5·7	
	(ii)	7·4	4·6	5·4
		7·3	4·6	5·6
Glycerophosphate ($0{\cdot}25\times10^{-1}$ M)	(i)	7·4	5·8	
		7·4	5·6	
	(ii)	7·4	4·6	6·4
		7·4	4·6	6·5
Orthophosphate (10^{-2} M)	(i)	7·5	5·2	
		7·5	5·2	
	(ii)	7·3	4·6	6·4
		7·3	4·6	6·3

Table 3 shows that this was not the case. As to the metaphosphate it is obvious that the buffering effect is very small compared with the inhibition of acid formation caused by the presence of this phosphate during incubation. In the pyrophosphate samples inhibition seems to be due mainly to the buffer effect. In the saliva–sucrose

mixtures supplemented with glycero- and orthophosphate there seems to be an antagonistic relation between buffer effect and effect on acid formation. The pH drop in the control samples (which dropped to 4·6) is reversed by post-incubational addition of orthophosphate to a pH of 6·4 and 6·3, whereas the pH dropped to 5·2 in the samples with orthophosphate present from the beginning of the incubation period. A similar stimulation of acid formation was found in the saliva–sucrose mixtures with glycerophosphate. As to the stimulation by orthophosphate, this observation is in accordance with the finding of LILIENTHAL (1956) who reports that addition of 5×10^{-3} M orthophosphate to salivary sediment slightly increased the rate of acid formation from sucrose.

TABLE 4. GELATINE DECALCIFICATION ASSAY

Intact human molars incubated at 37°C for 6 days in 50 ml of 2% gelatine solution. To these solutions phosphates (2%) and different amounts of 1·0 M lactic acid to yield a pH of 4 had been added. The macroscopic changes of the enamel surfaces observed after 6 days' acid gelatine decalcification are described in the last column (e). Column (a): initial pH of gelatine–phosphate solutions, (b): pH of gelatine–phosphate solutions after addition of 0·4 ml of 1·0 M lactic acid, sufficient to adjust unbuffered control gelatine solution (Treatment 5) to experimental pH of 4, (c): amount (ml) of lactic acid required to yield experimental pH of 4 in the gelatine solutions with different phosphates, (d): pH at end of experiment

Treatment	a	b	c	d	e
(1) Sodium metaphosphate	5·5	4·5	0·7	5·1	Severe etching (ca. 0·1 mm) of surface enamel. Surface rough, dull. No white spots.
(2) Sodium pyrophosphate	9·0	9·0	6·0	4·2	No alterations of enamel surface, no white spots.
(3) Di-sodium glycerophosphate	7·3	7·0	4·5	4·1	Surface intact, hard, shining. Extensive inner white spots, more pronounced than in control teeth.
(4) Sodium orthophosphate	7·4	7·0	4·0	4·3	Surface intact, white spots to same extent as in control teeth.
(5) Control	6·5	4·0	0·4	4·2	Surface intact, hard, shining. Characteristic white spots.

The observations obtained on the gelatine decalcification assay when 2% phosphates had been added are summarized in Table 4. Sodium pyrophosphate was highly effective in inhibiting white spot formation, whereas sodium metaphosphate severely etched and decalcified the enamel surface. The decalcification might be explained by the property of sodium hexametaphosphate of forming complexes with calcium:

$$Na_2\,[Na_4(PO_3)_6]+Ca^{\cdot\cdot}\longrightarrow Na_2\,[Na_2Ca(PO_3)_6]+2\,Na^{\cdot},\text{ and}$$

$$Na_2\,[Na_4(PO_3)_6]+2\,Ca^{\cdot\cdot}\longrightarrow Na_2\,[Ca_2(PO_3)_6]+4\,Na^{\cdot}.$$

The formation of complexes increases with lowering of the pH (STÜPEL, 1957), a fact which may explain the occurrence of severe etching in the acid gelatine solution

of pH 4 whereas there was no destructive effect on the teeth when the metaphosphate was administered in the diet dissolved in the saliva in the slightly alkaline milieu of the oral cavity.

In contrast to metaphosphate the ability of pyrophosphate to form complexes with calcium decreases with a fall in pH, which might explain the absence of white spot decalcifications in the pH 4 gelatine solution.

Sodium glycerophosphate seemed to favour the formation of white spots in acid gelatine.

DISCUSSION

In early investigations into the problem of phosphates and caries, the influence of dietary phosphorus and calcium on the mineral composition of the developing teeth was stressed. The present findings indicate a pronounced local effect of phosphates on fully developed teeth. The importance of local factors is supported by the findings of McCLURE and MULLER (1959b), according to which sodium orthophosphate is less cariostatic when administered by intubation than when added to the diet. In the same paper, concluding from their findings with mono-, di- and tribasic calcium phosphates, these authors suggest that rapid solubility in the oral cavity is a determining factor for a cariostatic action of phosphates. The fact that the insoluble calcium pyrophosphate has been found ineffective in reducing caries by BÜTTNER and MUHLER (1958) also points to the importance of solubility and local availability of the phosphate ions. These conclusions are contradictory to the theory of STRALFORS (1959) which postulates that insoluble phosphates become soluble and available when a fall in pH occurs at the enamel surface. However, animal experiments supporting this view were done using hamsters (STRALFORS, 1956) which more readily respond to minor experimental variables.

The lowered phosphorus removal rates in the metaphosphate and pyrophosphate groups in the present work are not conclusive as to the exact nature of possible alterations of the enamel surface, but they are considered to be an indication that some local changes have taken place which may be attributed to the action of the inhibitors on surface enamel. The calcium removal was not measured in this rat experiment, as it was preferred to investigate the calcium and phosphorus removal from the enamel by different decalcifying buffers in solutions containing different amounts of calcium and phosphorus on intact human teeth *in vitro* (MARTHALER, 1961). The results of this latter study have shown a close correlation between removal of phosphorus and calcium, confirming the findings of STRALFORS (1958). In an investigation into the effect of calcium and phosphate ions on the rate of enamel dissolution *in vitro*, STRALFORS (1959) found a reduction of the solution rate when the concentration of phosphate and calcium was increased.

The inhibition of acid formation found is in agreement with the findings of FOSDICK and KROB (1960) who observed such an inhibition in caries active saliva–sugar mixtures by meta- and pyrophosphates in a concentration between 50 and 100 mg per 100 ml of saliva.

The most pronounced anticariogenic effect was achieved with sodium pyrophosphate. Its caries-reducing potentiality was better than that of sodium orthophosphate, which inhibited the progression of lesions only (Table 1, Score B). However, considering the importance of the amount of phosphorus actually available, unrestricted comparability in the present experiment is limited to the metaphosphate and pyrophosphate groups, the diets of which were both supplemented with equal amounts of phosphorus (0·8 per cent). In the other groups, the phosphorus content was different. In another experiment (MÜHLEMANN, KÖNIG and MARTHALER, 1961) the caries inhibiting effects of disodium orthophosphate and sodium pyrophosphate supplemented to the diet at a level of 0·4 per cent phosphorus were quite similar. Taking into account the higher phosphorus level in the glycerophosphate group (0·29 per cent P), the superior cariostatic action of sodium orthophosphate (with only 0·17 per cent P) might be taken for a confirmation of the observations *in vitro* of OSBORN (1941), who found phosphate more effective in inorganic than in organic combination.

The effect of phosphoric acid—the only compound administered in the drinking water—is not directly comparable to the effects of phosphates administered in food. The apparent eroding effect was limited to tooth areas particularly exposed to mechanical friction by the tongue. In the absence of destructive acid effects on sulcal enamel, a cariostatic action in the fissures might have been possible. Such an effect was, however, not observed.

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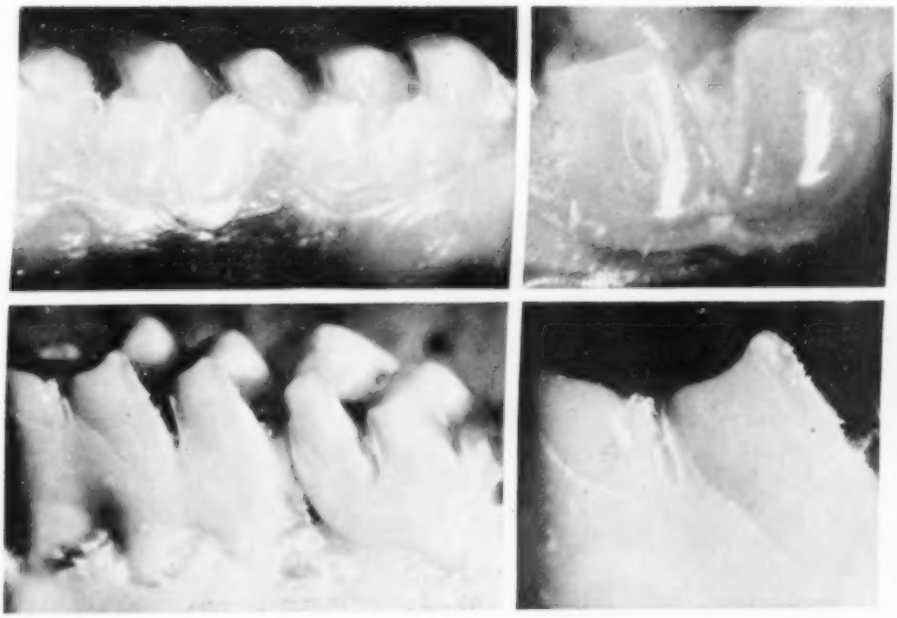

FIG. 2. Erosions on lingual surfaces of upper and lower molars in Osborne-Mendel rats at the end of a 20 day cariogenic period with diet 580 and 0·1% phosphoric acid in the drinking water. ×6 and ×10.

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THE EFFECT OF INJECTED MONOSACCHARIDES UPON THE MINERALIZATION OF RAT MOLARS

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Abstract—A solution containing glucose and fructose was injected intraperitoneally three times a day into rats for 10 days. Littermate controls received injections of physiologic saline. On the last day of the experiment, ^{32}P as orthophosphate was injected subcutaneously and immediately after the sugar and saline injections. The weights of the femora indicated that bone formation in the animals was slightly disturbed. In the partly erupted, whole first molar teeth and in the femora, the uptake of ^{32}P was slightly lowered by the injected sugar. The phosphate–carbonate ratio in the molar enamel of the experimental animals was slightly lower than in the controls.

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INTRODUCTION

NUMEROUS INVESTIGATIONS indicate that the cariogenic action of the carbohydrates is due to their acting as substrates for the production of acid on the tooth surface by oral micro-organisms. Furthermore, some evidence has been presented for the systemic action of carbohydrates during the pre-eruptive development of the teeth which subsequently becomes observable as an increased susceptibility of the experimental animals to dental caries. SOGNNAES (1948) showed that the offspring of hamsters, rats and mice became susceptible to dental caries when a high-sugar diet was fed to the dams during pregnancy and then continued during and after completion of the development of the teeth of the offspring. Subsequently STEINMAN and HALEY (1957a, b, c) and STEINMAN, HALEY and O'DAY (1958) gave sugars orally and by injection to rats during the pre-eruptive development of their molars and found they had become more susceptible to caries than their littermate controls. These workers also showed that carbohydrates differ in their ability to increase the subsequent caries. The nature of this systemic action may be a change in the oral environment of the teeth or in the developing teeth themselves.

HARTLES (1951) investigated the effect of a high-sugar cariogenic diet on the composition of continuously growing rat incisors. He found that the calcium and phosphorus content of the enamel and dentine of the experimental animals was higher than in control rats fed with a stock diet. The high-sugar diet had also lowered the calcium–phosphorus ratio of the enamel and the dentine. CREMER *et al.* (1953a) could find no changes of this kind in the molars of rats kept through many generations on a high-sugar diet. The uptake of radiophosphorus by the teeth of the "caries-animals", however, was higher than that of the controls (CREMER *et al.*, 1953b).

SOBEL and HANOK (1948, 1958) showed that the phosphate–carbonate ratio of the teeth of the rat is related to the calcium–phosphorus ratio of the food given to

the animals during tooth development. By providing a diet with a high calcium–phosphorus ratio, i.e. a low phosphorus diet, the carbonate content of the enamel and the dentine could be increased. Rats kept on such a diet during tooth formation were more susceptible to dental caries than control animals kept during that period on a diet with a low calcium–phosphorus ratio (SOBEL, 1952). The increased carbonate content of the teeth brought about by diet was induced by a lowered phosphate–carbonate ratio in the serum (SOBEL and HANOK, 1948). As glucose and fructose are known temporarily to lower the inorganic serum phosphorus, it seemed possible that similar changes in the composition of rat molars could be caused by the administration of large doses of sugar to the animals during the development of the molar teeth. It was thought by this means to give some explanation of the mechanism of the above mentioned systemic cariogenic action of sugar. A preliminary 1 day experiment indicated that the uptake of injected radiophosphorus by developing rat molars might be reduced by means of simultaneously injected glucose–fructose solution and gave rise to the experiments reported here.

MATERIALS AND METHODS

Twenty-seven 10 day old rats of three litters (Wistar strain) were divided by litters into two groups. One group, comprising seven males and seven females, was selected as an experimental group, the remaining seven males and six females serving as a control group.

Each animal of the experimental group received, in accordance with the method of STEINMAN *et al.* (1958) three intraperitoneal injections of sugar daily during a period of 9 or 10 days. According to MARSLAND (1952) enamel maturation in the first molar of the rat occurs within this age period. The injected solution contained 15 per cent glucose and 15 per cent fructose in physiologic saline solution, the quantity of sugar in one injection being 3·5 mg per gram of body weight. The control animals were given corresponding volumes of physiologic saline solution. Between injections the animals were nursed by their dams *ad libitum*. No unusual behaviour was observed in the experimental or control animals during the period of the injections. The dams were kept from 3 days before the experiment until its end on a diet which had the following percentage composition: casein, 22·0; corn starch, 51·0; brewer's yeast, 3·8; wheat germ, 2·0; butterfat, 1·6; lard, 3·1; liver concentrate, 1·6; olive oil, 1·6; salts, 5·5; water to moisten, 7·8; fresh lettuce; vitamin D_3 I.U., 4·0 per gram of diet. The calcium and phosphorus content of this diet were 1·35 and 0·88 per cent respectively. The drinking water was distilled from a low fluoride tap water.

The final experimental day for each litter was so chosen that the mean eruptive state of the upper first molars observed by means of an ear speculum was the same in each litter, four cusps being fully erupted.

On the last day, when the animals were 19–20 days old, they were finally removed from their dams early in the morning. After 5 hr each animal was weighed, and then was given approximately 1 ml of cow's milk by means of a stomach tube. One half hour later approximately 2 μc of radiophosphorus was given to each of the animals by an injection under the dorsal skin. The isotope solution with high

specific activity contained 5 μc of ^{32}P per millitre as orthophosphate in isotonic saline solution containing phosphate buffer, pH 7. The isotope injections were carried out with a centigrade 0·5 ml tuberculin syringe. Immediately following the isotope injection, each animal of the experimental group was given a sugar injection as mentioned above. The control animals received an injection of physiologic saline. All the administrations were repeated 4 hr later. The third doses of sugar and saline were injected 3 hr later, and after a further 3 hr the animals were decapitated.

The first and second molars and the right femora were removed and cleaned from the adhering soft tissues, including the pulps of the still rootless molars and the marrows of the bones. After a thorough brushing in running tap water and in distilled water, the teeth and bones were dried overnight at 105°C and weighed. This and the subsequent weighings were carried out with a balance having a sensitivity of 0·1 mg. After weighing the teeth and bones were powdered in a mortar and sifted through a 100 mesh screen. All the first molars of each animal were powdered together, the second molars being handled in the same way. Thus three different samples were obtained from each animal.

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For the radioactivity measurement, the samples were redried. Then 12±0·3 mg of each sample was weighed, and placed in an aluminium cup in a layer of even thickness. The radioactivity of the samples and the injected solution, as diluted and dried, was measured by means of a Geiger–Müller tube connected to an automatic counter.

For the chemical analyses, the powders of the first molars were kept in a mixture of alcohol and ether overnight. In order to obtain sufficient quantities of material for the final separated samples, the powders from two animals were pooled. Separation of the enamel and the dentine was carried out in accordance with the method of MANLY and HODGE (1939). Enamel of 98 per cent purity was obtained at a density of 2·6 of the separation fluid. The density for 99 per cent pure dentine was 2·5. After two washings in acetone and drying, the samples were weighed twice. The average amounts of enamel and dentine obtained were 8·8 and 14·9 mg respectively in the experimental group, the corresponding amounts of the control group being 10·2 and 18·9 mg.

The carbon dioxide content of both the enamel and the dentine was determined by the Sobel modification (SOBEL, ROCKENMACHER and KRAMER, 1944) of the van Slyke–Folch manometric method (VAN SLYKE and FOLCH, 1940), making use of the reagents and factors of MCFADYEN (1942). The phosphorus content was determined in duplicate by the FISKE–SUBBAROW method (1925) making use of a photo-electric spectrophotometer. The calcium content was determined in duplicate by precipitation as oxalate and by titration with potassium permanganate.

RESULTS

The results of different weight determinations can be seen in Table 1.

The relative weight gain by both the experimental and the control animals during the 10 days experimental period was the same. The femora of the experimental animals, calculated as a percentage of the final weight of the animal, were lighter

than the femora of the control animals. A similar, but non-significant tendency was observed in the molars.

TABLE 1. THE WEIGHTS OF THE ANIMALS AND THEIR DRIED HARD TISSUES IN RELATION TO INJECTED SUGAR. MEAN VALUES

Group	No. of animals	Weight of animals (g)		Weight gain (g)	Weight gain (%)	Molars		Femur (mg)	Femur per cent of final weight
		Initial	Final			First (mg)	Second (mg)		
Experimental	14	18·4	32·3	13·9	75·5	7·3	4·5	40·9	0·126 ± 0·011*
Control	13	18·7	32·8	14·1	75·5	7·5	4·8	43·9	0·134 ± 0·008
									$P < 0·05$

* Standard deviation.

The radioactivity obtained for each sample was corrected for background and decay, and calculated as a percentage of the injected dose per 100 mg of dried tissue. These results are given in Table 2. The uptake of radiophosphorus by the whole, partly erupted first molars and femora of the experimental animals was significantly lower during the 10 hr experimental period than that of the control rats. In the unerupted second molars, the difference was not significant.

TABLE 2. THE EFFECT OF INJECTED SUGAR ON THE UPTAKE OF RADIOPHOSPHORUS BY THE MOLARS AND FEMORA OF RATS. MEAN VALUES ± STANDARD DEVIATION

Group	No. of animals	Per cent of injected dose per 100 mg of tissue		
		First molar	Second molar	Femur
Experimental	14	1·59 ± 0·25	3·40 ± 0·81	6·22 ± 1·03
Control	13	1·87 ± 0·26	3·83 ± 0·76	7·08 ± 1·01
P		<0·01	—	<0·05

The results of the chemical investigations are shown in Table 3. The calcium–phosphate ratio in the enamel of the experimental animals was higher than the corresponding ratio of the control animals. This difference, however, was not significant. The phosphate–carbonate ratio in the enamel of the experimental animals was significantly lower than that of the control animals. No significant differences were found in the dentine between the experimental and the control animals. However, the differences seem to exhibit the same tendency as the differences in the enamel.

TABLE 3. THE EFFECT OF INJECTED SUGAR ON THE COMPOSITION OF THE ENAMEL AND DENTINE OF RAT MOLARS. MEAN VALUES ± STANDARD ERROR OF THE MEAN

Group	No. of samples	% Ca	% P	% CO_2	Ca/PO_4	PO_4/2CO_2
Enamel						
Experimental	6	33·5	15·7	2·27	1·65 ± 0·06	5·49 ± 0·19
Control	6	32·3	16·2	2·05	1·54 ± 0·09	6·35 ± 0·18
P					—	< 0·01
Dentine						
Experimental	6	27·8	12·5	1·80	1·72 ± 0·08	5·54 ± 0·17
Control	6	26·6	12·8	1·79	1·61 ± 0·06	5·74 ± 0·19

The Ca/PO_4 and PO_4/2CO_2 values denote molar ratios.

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DISCUSSION

The results given above show that the formation of the hard tissues may be influenced by the injection of glucose and fructose during the period of formation.

The same relative weight gain by both the experimental and control animals indicates that no marked increase in the formation of fat occurred as a consequence of the sugar injections. Thus the relatively lighter femora of the experimental animals may signify a disturbed bone formation.

The decreased uptake of radiophosphorus by the hard tissues of the experimental animals might indicate a reduction in the mineralization process without changes in the relations of phosphorus to the other depositing tissue components, or a replacement of phosphate by other anions. The chemical investigations show that the second alternative might occur in the enamel. This change in the composition of the enamel is similar to though not as intense as that caused by lowering the phosphorus content of the diet (SOBEL and HANOK, 1948, 1958).

The most likely factor in bringing about the results obtained in the present study is the effect of sugars in temporarily lowering the inorganic serum phosphorus level. The rapid formation of carbon dioxide as an end product of the monosaccharide metabolism might also contribute slightly to a fall in the phosphate–carbonate ratio in the serum. This temporary fall could then bring about a lowering of the ratio between the phosphate and carbonate ions surrounding the calcifying tissues. When repeated this could, in turn, produce detectable variations in the enamel. The tendency of the pH of tissue fluids to decrease as a consequence of sugar metabolism is generally well controlled, and thus an increased adsorption of carbonate could occur.

The present results exhibit a tendency the reverse of that found by HARTLES (1951) in respect of the effect of a high sugar diet on the phosphorus content of rat incisors.

The present results may in part provide an explanation of the increased susceptibility to caries apparently induced by the systemic action of monosaccharides noted by STEINMAN *et al.* (1958). According to SOBEL (1952), decreased phosphate–carbonate ratio in the teeth is correlated with increased susceptibility to caries. This finding is furthermore in accord with work which shows the preferential solution of the enamel carbonate when it is exposed to acids (LOGAN and TAYLOR, 1938; HARDWICK, 1949; GORE, 1953; COOLIDGE and JACOBS, 1957) and with the increased solubility of calcium phosphate (GREENWALD, 1945) and hydroxyapatite (ERICSSON, 1952) in the presence of carbonate.

Further studies will be devoted to the more important problem of whether sugar, given via the alimentary tract, can have the same effect on tooth composition as that outlined above, and the possibilities of reducing this effect.

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THE CARBOHYDRATES OF HUMAN PAROTID SALIVA

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Abstract—The free and protein-bound carbohydrates in human parotid saliva were identified and assayed. The only free carbohydrate found was glucose (200γ/100 ml). The bound carbohydrates include hexosamines, methylpentose, hexoses, sialic acid and deoxyribose. Based on analyses of samples from forty-three persons in the 20–25 year age group, the quantities of these, per 100 mg of salivary protein, were: hexosamines, 4·5 mg; methylpentose, 3·6 mg; galactose + mannose, 13·6 mg; glucose, 0·5 mg; sialic acid, 0·11 mg; deoxyribose, 0·15 mg.

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RECENT STUDIES on the composition of parotid saliva using paper electrophoresis indicated that some of the proteins which were detected had significant quantities of carbohydrate associated with them (DREVON and DONIKIAN, 1956; FERGUSON, KRAHN and HILDES, 1958; GABL and EGGER, 1959; HARNDT, 1960; MANDEL and ELLISON, 1961). Small quantities of free carbohydrates have also been found (PIGMAN and HAWKINS, 1958; SHANNON and PRIGMORE, 1960).

As a part of a continuing study of the relation between the composition of human saliva and oral disease, determinations were made of the nature and amount of carbohydrates in parotid saliva. These data are presented below.

MATERIALS AND METHODS

Collection of samples

Stimulated parotid saliva was collected from forty-three persons between the ages of 20 and 25 years, using Lashley cups as modified by Stephan. The samples used for identification of carbohydrates were shell-frozen in dry-ice–alcohol and then lyophilized, either immediately or after several days storage at —20°C. The dried powders were kept in the refrigerator until they were analysed. The samples used for assay were stored in the freezing compartment of the refrigerator until used.

Identification of carbohydrates

The lyophilized powder from a 60 ml sample was suspended in a small volume of water, placed in a dialysis membrane, and dialysed against 100 ml of water at 4°C for 48 hr with constant stirring. The dialysate, which contained the non-protein-bound or "free" carbohydrates was passed through a mixed resin column (Amberlite MB-3) and the eluate concentrated and analysed by a modified reducing sugar test (SCHIFFMAN, HOWE and KABAT, 1958), the primary cysteine reaction (DISCHE, 1955)

and the glucose oxidase reaction (KEILIN and HARTREE, 1948). (The glucostat reagents prepared by Worthington Biochemical Corp. Freehold, N.J., were used.)

For the analysis of protein-bound neutral sugars, the lyophilized powder from 100 ml of parotid saliva was hydrolysed with Permutite Q (GLEGG, EIDINGER and LEBLOND, 1953). The hydrolysate was passed through Amberlite MB-3, the eluate concentrated and (a) analysed for methylpentose and total hexose by the primary cysteine reaction and for glucose with the glucose oxidase reaction, (b) chromatographed on paper using propanol:ethyl acetate:water (7:1:2) or propanol:ethanol:water (7:1:2) quadruple descent and developed with aniline hydrogen phthalate or with aniline oxalate (BLOCK, DURRUM and ZWEIG, 1958); and (c) separated on phosphate-impregnated glass fibre sheets (GABRIEL and IGALS, 1960) using the propanol:ethyl acetate:water as solvent and the same developers as for paper. In addition, chromatograms on glass fibre sheets were developed with glucose oxidase as described by SALTON (1960).

Confirmatory evidence for the identity of the monosaccharides was obtained after eluting the separated substances from the glass fibre sheets. Each eluate was analysed for total reducing sugar. Subsequent tests with the primary and secondary cysteine reactions, and comparison with the reducing sugar values, enabled the distinction between glucose, galactose, mannose and fucose to be made (DISCHE, 1955).

To separate the hexosamines, lyophilized saliva was first hydrolysed with 2 N HCl for 2 hr. The hydrolysate was dialysed against water, the dialysate concentrated and chromatographed on a Dowex-50 column (GARDELL, 1953) using 0·3 N HCl for elution, at a flow rate of 1·5–3 ml/hr, collecting 0·5 ml per tube. The eluates were assayed for reducing substances and the separated fractions analysed for glucosamine and galactosamine (SCHIFFMAN *et al.*, 1958).

Assay of carbohydrates

Samples were thawed and dialysed against water for 48 hr at 4°C to remove non-protein-bound material. Fucose and hexose were measured by the primary cysteine reaction and hexosamine by a modified Elson–Morgan reaction (MANDEL and ELLISON, 1961). Several samples were also hydrolysed with 2 N HCl for 2 hr and analysed for glucose by the glucose oxidase method, and for deoxyribose by the thiobarbituric acid reaction (WARAVDEKAR and SASLAW, 1957). Sialic acid was determined after hydrolysis with 0·1 N HCl at 80°C for 45 min using the thiobarbituric acid assay of WARREN (1959). Protein was estimated with the Folin–Ciocalteau phenol reagent (KABAT and MAYER, 1948). A tyrosine solution was employed as the colorimetric standard.

RESULTS

Identification of the carbohydrates

Analysis of the dialysates prepared from parotid saliva disclosed the presence of about 0·2 mg per cent of dialysable sugar. Comparison of the results obtained using the reducing sugar test and the primary cysteine and glucose oxidase reactions indicated that all the dialysable sugar was glucose.

The chromatograms of hydrolysates revealed the presence of three neutral sugars, namely galactose, mannose and fucose (Fig. 1). Comparison of primary and secondary cysteine and reducing sugar values of eluates from glass fibre sheets corroborated the identifications. When the hydrolysate was assayed with the glucose oxidase test, the reaction indicated that only about 2 per cent of the total hexose was glucose. This small amount of glucose could not be detected on chromatograms stained with aniline hydrogen phthalate or aniline oxalate. Chromatograms on glass fibre sheets developed with glucose oxidase showed a faint spot with the appropriate R_f.

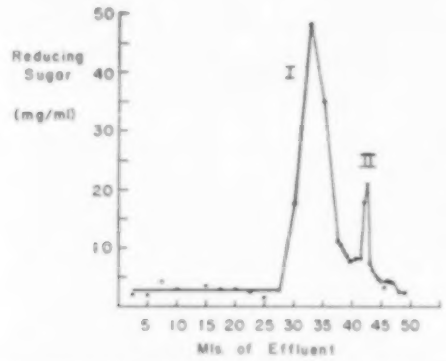

FIG. 2. Chromatography of acid-hydrolysed parotid saliva on Dowex-50. (Eluted with 0·3 N HCl; 0·5 ml/tube).

Chromatography on Dowex-50 enabled the separation of two reducing substances (Fig. 2). These were identified after acetylation as being glucosamine and glactosamine. Of the total amino-sugar, 85 per cent consisted of the former and 15 per cent of the latter substance.

TABLE 1. THE PROTEIN-BOUND CARBOHYDRATES OF HUMAN PAROTID SALIVA

	No. of subjects	mg/100 ml			mg/100 mg protein		
		Average	S.D.	Range	Average	S.D.	Range
Protein	—	165·2	58·3	56·0–295·0			
Hexosamine	43	7·5	3·3	2·0–15·2	4·5	1·2	1·2–7·8
Methylpentose	43	5·9	2·6	1·5–12·9	3·6	1·0	0·9–6·3
Galactose+ mannose	43	22·9	10·3	4·7–47·6	13·6	5·6	6·5–34·9
Sialic acid	13	0·27	*	0·11–0·36	0·11	*	0·10–0·14
Deoxyribose	11	0·19	*	0·17–0·22	0·15	*	0·09–0·29
Glucose	4	1·5	*	1·0–2·6	0·5	*	0·3–0·7

* Not calculated.

Assay of the carbohydrates

The data given in Table 1 describe the concentrations in saliva of the several protein-bound carbohydrates. It is of interest that when the concentrations were calculated in relation to the protein content of the sample the ratio of the standard deviation to the mean of the fucose and hexosamine values were appreciably smaller than when a volume basis was employed. This was not true of the hexose.

DISCUSSION

It is apparent that despite the serous character of the parotid gland, its secretion contains a significant amount of carbohydrate, the bulk of which is associated with the proteins. Other workers have noted the presence of a small amount of free sugar, but did not identify it. The present evidence indicates that it is all glucose. Glucose values similar to those we found were very recently reported by SHANNON, PRIGMORE and BROOKS (1960).

In the only recent studies on the protein-bound reducing substances of parotid saliva (GORE, 1956; PIGMAN and HAWKINS, 1958), the average concentrations reported (as "glucose") were somewhat lower than those which we have found. This may have been a consequence of the differences in experimental methods, or of the use of glucose as a standard in colorimetric procedures. Based on our findings, it appears preferable to employ a standard consisting of equal amounts of galactose and mannose or to apply a correction to determinations made with a glucose standard.

There are no previous reports with respect to fucose or hexosamines in parotid saliva. SCHNEYER (1956) was unable to detect sialic acid in parotid saliva using the direct Ehrlich reaction. The thiobarbituric acid assay which we employed is much more sensitive and enabled the measurement of the small amount of sialic acid actually present.

It should be noted that the data described here are based entirely on persons in the 20–25 year old range. There may be differences in the secretion at other ages. Fucose and hexosamine determinations provide a measure of the glycoprotein in parotid saliva since almost all the fucose and most hexosamine are part of the two glycoproteins (MANDEL and ELLISON, 1961). Such measurements should provide useful additional parameters to employ in studying the physiology of salivary secretion and the relation of salivary composition to oral disease.

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Note added in proof: While this manuscript was in press, ZIPKIN, MERGENHAGEN and KASS reported measurements of the sialic acid content of whole saliva and parotid saliva (*Biochem. biophys. Res. Comm.* **4**, 76, 1960) employing the same analytical procedure that we used. They found that the average concentration of sialic acid in the parotid saliva of eight normal young adults was 2·4 mg per cent.

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Because of the discrepancy between their values and ours we rechecked our technique and found an error which was responsible for the destruction of a considerable portion of the sialic acid. On analysis of twelve additional samples of parotid saliva using the corrected technique we found an average of 1·77 mg of sialic acid per 100 ml of saliva and an average of 0·98 mg per 100 mg of protein.

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FIG. 1. Chromatograph of parotid saliva hydrolysed with Permutit Q. (Propanol: ethanol: water, 7:1:2, quadruple descent developed with aniline oxalate). FUC = fucose; MAN = mannose; GAL = galactose; GLU = glucose; St. = standards; P.S. = parotid saliva hydrolysate.

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THE EFFECT OF CALCIUM HYDROXIDE AND AMALGAM ON NON-CARIOUS, VITAL DENTINE

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Abstract—Twenty-five non-carious teeth from children and young adults have been used in this study to determine the effect of calcium hydroxide and amalgam on dentine. Cavity preparations were made and the material to be tested was placed on the pulpal wall. A few teeth were left unoperated as controls. The teeth were extracted after varying lengths of time. Microhardness tests have been the principal method of investigation of tissue changes in the dentine. Further information has been obtained by densitometric evaluation of mieroradiographs. The results indicate a highly significant increase in mineralization of calcium hydroxide covered dentine. Little or no change takes place in the amalgam covered dentine.

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INTRODUCTION

THE effect of restorative materials used in dentistry has to a large extent been limited to studies on the dental pulp. Possible changes in the primary dentine have been investigated less extensively. SOWDEN (1956) indicated that soft carious dentine covered by calcium hydroxide will remineralize. A white line in the dentine under the calcium hydroxide in postoperative radiographs supported this clinical observation. KLEIN (1958) reported an increase in the radiopacity of the primary dentine of teeth previously restored with a base of calcium hydroxide in a methyl cellulose suspension. A densitometric evaluation of postoperative intra-oral roentgenograms indicated a marked increase in mineralization of the area of dentine between the calcium hydroxide and the pulp. The author pointed out, however, that some of the recorded changes in the dentine probably were due to a reaction of the dentine to the carious attack as well as possible changes due to cavity preparations and the insertion and maintenance of the restoration in the tooth.

ZANDER (1959) has indicated that calcium hydroxide should always be placed between dentine and cement to lessen the injurious effect on the pulp. Any possible effect on the primary dentine was, however, not taken into consideration.

The effect of amalgam on dentine has been studied by MASSLER and BARBER (1953). A discoloration was noticed in 85 per cent of the teeth examined. The discoloured dentine was radiopaque, did not bleach with "peroxide" and the tubular structure was preserved. A galvanic action was suggested as the mechanism whereby metallic ions were precipitated in the dentine. Possible tissue changes within the dentine did not appear to be considered as causing part of the observed radiopacity. An experiment *in vitro* seemed to support the theory of precipitation of metallic ions.

This study was undertaken to further investigate changes in young, primary dentine by the use of calcium hydroxide and amalgam in cavity preparations.

MATERIALS AND METHODS

Twenty-five teeth from a total of ten patients were used in this study. Seventeen of these were premolars, in children, which were to be extracted for orthodontic reasons. Seven were third molars and one was a supernumerary (fourth) molar in young adults which were to be extracted due to malposition or lack of space in the dental arches. The average age of the children was 11·5 years and of the adults 24·0 years.

The teeth selected were all clinically caries-free. In two of the cases where third molars were used, the occlusal fissures showed marked staining, and this could possibly indicate an early carious attack. The lingual surfaces were therefore used for the experiment.

Single surface cavity preparations of varying depths were cut in the experimental teeth without using a local anaesthetic. Occlusal preparations were used in most cases, but some were prepared on the lingual or buccal surfaces. Great care was taken in the preoperative examination of the surfaces to exclude any that showed evidence of early carious attack. One tooth was left unoperated in cases where more than two teeth were available.

A high speed dental engine with water spray and sharp carbide burs were used in the preparation of the cavities. The prepared cavities were dried gently with cotton and warm air, taking care not to dry the preparation excessively. Of the twenty-five teeth used, eighteen received experimental restorations and seven were left unoperated. Four of the eighteen teeth in the experimental group received two restorations. A total of twenty-two restorations have therefore been used in this study. Sixteen of these were restored with a calcium hydroxide base and amalgam, and six with amalgam only.

Immediately after the extraction of the teeth, the enamel was partially removed with a diamond stone under an abundant water spray to make the subsequent sectioning easier. The teeth were then embedded in Ward's Bio-Plastic.

The teeth were sectioned with the Gillings Hamco Thin Sectioning Machine (GILLINGS and BUONOCORE, 1959). The restored teeth were first bisected longitudinally through the approximate midpoint of the restoration. Cross-sections of these bisected teeth were then cut at different levels between the pulpal wall of the restoration and the pulp (Figs. 1 and 2). The unoperated teeth were sectioned in a similar manner (Fig. 3). Three or more surfaces of the embedded teeth exposed by the sectioning were tested with the Kentron Micro Hardness Tester. A 50 g load was used with the indenter resting on the specimen for 15 sec, and the indentations were read through a 20× objective. Microhardness tests were done on the area of dentine between the base of the restoration and the pulp as well as on areas remote from the restoration. Comparable areas were tested in the unoperated control teeth (Figs. 1–3). Microhardness is expressed in Knoop Hardness Numbers (K.H.N.).

A densitometric evaluation was made of ten microradiographs of sections of dentine. Again particular emphasis was placed on the comparison of restoration covered dentine and unoperated control dentine. This part of the study has been

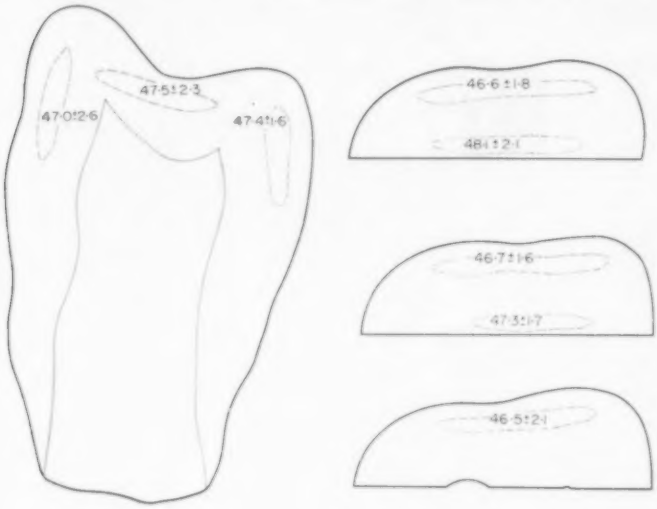

FIG. 1. Case 1 (S.S.)—Areas tested by microhardness. Unoperated tooth. K.H.N. "unaffected" dentine 47·2±2·1.

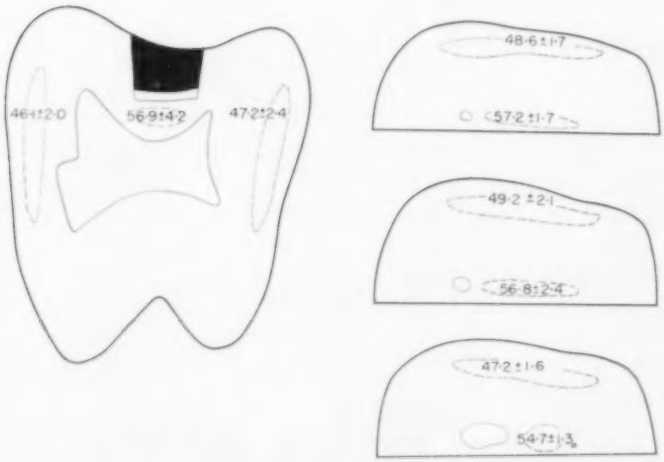

FIG. 2. Case 1 (S.S.)—Areas tested by microhardness. Tooth has an amalgam restoration with a calcium hydroxide base.

K.H.N. $Ca(OH)_2$ covered dentine	56·3±2·8
K.H.N. "unaffected" dentine	47·6±2·3
Mean difference	8·7

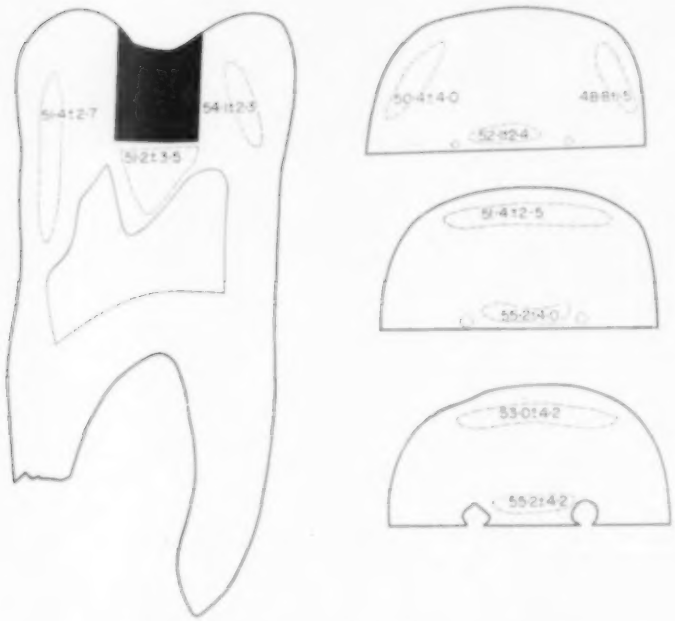

FIG. 3. Case 2 (S.G.)—Areas tested by microhardness. Tooth has an amalgam restoration.

K.H.N. Amalgam covered dentine	52·7±5·6
K.H.N. "unaffected" dentine	51·5±3·1
Mean difference	1·2

limited to a comparison of two microradiographs of sections from the same tooth of approximately the same thickness and with the same exposure and development time. The densitometric readings were done on selected areas with the low scale of a Photovolt densitometer. The thickness of these areas was then measured in microns with a Federal dial gauge supplied with a point contact. The density reading was divided by the thickness for the same area, and the change in density per micron was recorded.

RESULTS

Examples of the various surfaces investigated by microhardness testing are illustrated in Figs. 1–3. The term "unaffected" dentine is used to describe fully formed dentine in unoperated teeth as well as dentine areas remote from the experimental restorations. It is interesting to note that the newly erupted teeth used in this experiment showed very little variation in the microhardness of coronal dentine in unoperated control teeth (Fig. 1). Calcium hydroxide-covered dentine does, however, show a marked increase in microhardness (Fig. 2). It is also apparent that there is

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no significant difference in the microhardness of "unaffected" dentine in these two teeth from the same patient. The mean difference between amalgam-covered dentine and "unaffected" dentine (Fig. 3) is not statistically significant.

TABLE 1. MICROHARDNESS OF $Ca(OH)_2$ COVERED DENTINE AND "UNAFFECTED" DENTINE IN THE SAME TOOTH

Case No.	Restoration *in situ* (days)	K.H.N. $Ca(OH)_2$ covered dentine ($m \pm$S.D.)	K.H.N. "unaffected" dentine ($m_1 \pm$S.D.)	Mean difference ($m - m_1$)
1	15	56·3±2·8	47·6±2·3	8·7*
8	50	58·6±3·3	47·4±1·7	11·2*
3	56	58·3±2·0	47·8±2·8	10·5*
3	56	62·2±9·4	47·4±2·2	14·8*
3	56	62·4±4·6	47·9±1·8	14·5*
6	59	55·7±3·2	46·3±2·0	9·4*
5	60	58·8±4·2	48·5±3·5	10·3*
5	75	56·5±4·9	48·9±2·2	7·6*
9	71	56·9±2·0	46·4±1·8	10·5*
7	74	55·0±2·7	48·9±3·0	6·1*
7	112	57·6±1·9	47·6±1·5	10·0*
7	112	57·3±2·2	47·9±1·6	9·4*
4	92	55·8±3·1	48·1±1·9	7·7*
4	92	54·0±2·5	47·9±2·2	6·1*
10	94	57·9±1·9	46·4±1·5	11·5*
2	139	67·6±10·3	50·2±3·0	17·4*

* $P < 0.001$.

TABLE 2. MICROHARDNESS OF $Ca(OH)_2$ COVERED DENTINE IN OPERATED TOOTH AND "UNAFFECTED" DENTINE IN UNOPERATED CONTROL TOOTH OF SAME PATIENT

Case No.	Restoration *in situ* (days)	K.H.N. $Ca(OH)_2$ covered dentine (operated tooth) ($m \pm$S.D.)	K.H.N. 'unaffected" dentine (unoperated control tooth) ($m_1 \pm$S.D.)	Mean difference ($m - m_1$)
1	15	56·3±2·8	47·2±2·1	9·1*
8	50	58·6±3·3	47·9±2·4	10·7*
3	56	58·3±2·0	48·6±3·6	9·7*
3	56	62·2±9·4	48·6±3·6	13·6*
3	56	62·4±4·6	48·6±3·6	13·8*
5	60	58·8±4·2	49·5±4·4	9·3*
5	75	56·5±4·9	49·5±4·4	7·0*
7	74	55·0±2·7	49·2±2·7	5·8*
7	112	57·6±1·9	49·2±2·7	8·4*
7	112	57·3±2·2	49·2±2·7	8·1*
4	92	55·8±3·1	48·6±2·4	7·2*
4	92	54·0±2·5	48·6±2·4	5·4*
10	94	57·9±1·9	46·7±2·1	11·2*

* $P < 0.001$.

Table 1 summarizes the microhardness results of calcium hydroxide-covered dentine and "unaffected" dentine in the same tooth. A statistically highly significant increase in microhardness of calcium hydroxide-covered dentine was found in all cases. The same is also apparent if the microhardness of calcium hydroxide-covered dentine and "unaffected" dentine from an unoperated control tooth of the same patient is compared (Table 2).

TABLE 3. MICROHARDNESS OF AMALGAM-COVERED DENTINE AND "UNAFFECTED" DENTINE IN THE SAME TOOTH

Case No.	Restoration *in situ* (days)	K.H.N. amalgam covered dentine (m±S.D.)	K.H.N. "unaffected" dentine (m_1±S.D.)	Mean difference ($m-m_1$)	*P*
3	56	48·4±2·0	47·8±2·6	0·6	<0·4
3	56	47·9±1·9	47·4±2·2	0·5	<0·4
7	74	49·1±1·9	48·9±3·0	0·2	<0·8
7	112	47·7±1·7	47·9±1·6	−0·2	<0·8
4	92	50·1±3·0	48·0±2·6	2·1	<0·001
2	139	52·7±5·6	51·5±3·1	1·2	<0·2

The relationship between the microhardness of amalgam-covered dentine and "unaffected" dentine in the same tooth is in Table 3. Case 7 showed a slight decrease in microhardness of amalgam-covered dentine, which is not statistically significant. Case 4 indicated a statistically highly significant increase in microhardness of amalgam-covered dentine, but with fewer readings it is apparent that the mean difference is too small to be significant with the rather large standard deviations.

TABLE 4. MICROHARDNESS OF AMALGAM-COVERED DENTINE IN OPERATED TOOTH AND "UNAFFECTED" DENTINE IN UNOPERATED CONTROL TOOTH OF SAME PATIENT

Case No.	Restoration *in situ* (days)	K.H.N. amalgam covered dentine (operated tooth) (m±S.D.)	K.H.N. "unaffected" dentine (unoperated control tooth) (m_1±S.D.)	Mean difference ($m-m_1$)	*P*
3	56	48·4±2·0	48·9±3·6	−0·5	<0·5
3	56	47·9±1·9	48·9±3·6	−1·0	<0·05
7	74	49·1±1·9	49·2±2·7	−0·1	<0·8
7	112	47·7±1·7	49·2±2·7	−1·5	<0·005
4	92	50·1±3·0	48·6±2·4	1·5	<0·005

Table 4 shows the differences in microhardness of amalgam-covered dentine and "unaffected" dentine in an unoperated control tooth of the same patient. Again it is apparent that the difference between the two is small for all cases, and that there actually are indications to suggest a decrease of microhardness of amalgam-covered dentine as compared with dentine in an unoperated control tooth.

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Where two restorations were used in the same tooth, the differences in the microhardness are presented in Table 5.

TABLE 5. MICROHARDNESS OF $Ca(OH)_2$ COVERED DENTINE, AMALGAM COVERED DENTINE AND "UNAFFECTED" DENTINE IN THE SAME TOOTH

Case No.	Restoration *in situ* (days)	K.H.N. $Ca(OH)_2$ covered dentine (m±S.D.)	K.H.N. amalgam covered dentine (m±S.D.)	K.H.N. "unaffected" dentine (m_1±S.D.)	Mean difference ($m-m_1$)	*P*
3	56	58·3±2·0	—	47·8±2·6	10·5	<0·001
		—	48·4±2·0	47·8±2·6	0·6	<0·4
	56	62·2±9·4	—	47·4±2·2	14·8	<0·001
		—	47·9±1·9	47·4±2·2	0·5	<0·4
7	74	55·0±2·7	—	48·9±3·0	6·1	<0·001
		—	49·1±1·9	48·9±3·0	0·2	<0·8
	112	57·3±2·2	—	47·9±1·6	9·4	<0·001
		—	47·7±1·7	47·9±1·6	−0·2	<0·8

The area of increased hardness between the calcium hydroxide base and the predentine was in all cases well limited. The microhardness tests showed a sudden increase, if continued, along a straight line on a bisected tooth between the calcium hydroxide base and the pulp (Fig. 4).

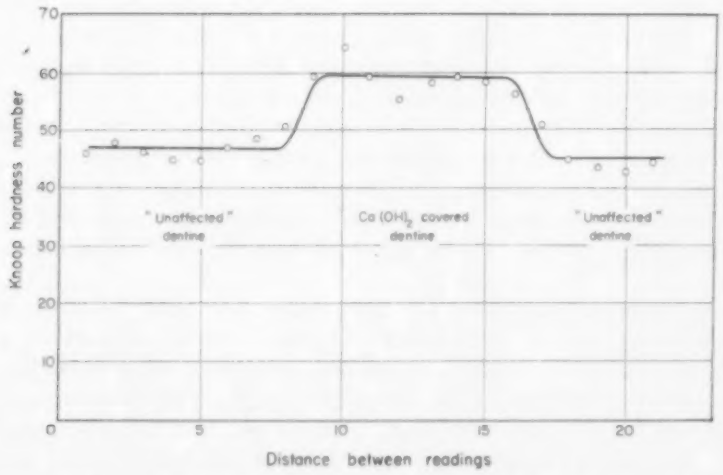

FIG. 4. Case 8 (A.S.)—Hardness results showing the relationship between Knoop hardness number and the distance between the readings.

The percentage increase in X-ray absorption, as revealed by the density evaluation of the microradiographs, appears to have a linear relationship to the percentage increase in microhardness (Fig. 5). The three cases showing the most marked increase in X-ray absorption are cases of calcium hydroxide-covered dentine. The two cases with a relatively small increase are cases of amalgam-covered dentine.

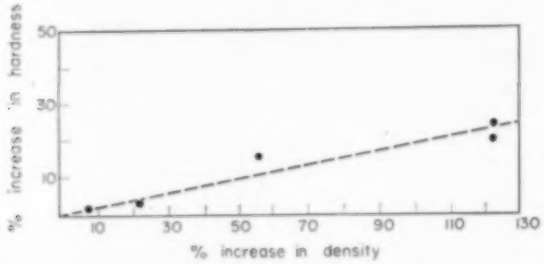

FIG. 5. Relationship between increase in hardness and increase in density.

DISCUSSION

The microhardness results obtained in this study have conclusively shown that young, non-carious, vital dentine, covered by a mixture of calcium hydroxide and water, shows a marked and statistically highly significant increase in hardness as related to "unaffected" dentine in the same tooth as well as "unaffected" dentine from unoperated teeth in the same patient (Tables 1 and 2). The effect of the length of time the calcium hydroxide covers the dentine is not clear. This study does not with any certainty suggest that the longer the calcium hydroxide is in place in the tooth, the harder the dentine becomes. The time required to produce the change is relatively short, and this study shows that a marked increase can be recorded after 15 days. Individual susceptibility of each tooth to produce a change seems to vary considerably, and any time factor involved may be overshadowed by this.

The depth of the cavity preparation in which the calcium hydroxide was placed did not appear to have a noticeable effect on the increase in hardness. The difference in hardness recorded has not correlated with the depth of the preparation.

The effect of the preparation, insertion and maintenance of restoration in the teeth appears to be very small. These factors together with a possible specific effect of amalgam may account for the minor changes recorded in amalgam-covered dentine.

The densitometric evaluation of microradiographs of dentine sections supported the microhardness findings. The results obtained by these two methods are interpreted as an expression of increased mineralization of calcium hydroxide-covered dentine. MJOR (1960a) has used the term "secondary intradentinal mineralization" to describe this change. Again it became apparent that the amalgam-covered dentine showed only a small change compared to calcium hydroxide-covered dentine. In this experiment no discoloration was observed in the amalgam-covered dentine in any of the cases.

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This study does not reveal the nature and process involved in the apparent increase in mineralization of calcium hydroxide-covered dentine. Histological studies of ground sections (MJOR, 1960b) have, however, suggested that the increase in mineralization to a large extent can be accounted for by additional mineral deposition in the walls of the dentinal tubules (secondary intratubular mineralization).

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